

SHUTTLE AGENT PEPTIDES OF MINIMAL LENGTH AND VARIANTS THEREOF ADAPTED FOR TRANSDUCTION OF CAS9-RNP AND OTHER NUCLEOPROTEIN CARGOS

The present description relates to the intracellular delivery of nucleoprotein cargoes via peptide-based delivery systems. More specifically, the present description relates to the use of synthetic peptide shuttle agents for the intracellular delivery of nucleoprotein cargoes such as Cas9-RNPs, as well as synthetic peptide shuttle agents engineered for increased resistance to inhibition by nucleoproteins and/or extracellular DNA/RNA.

The present description refers to a number of documents, the contents of which are herein incorporated by reference in their entirety.

BACKGROUND

Genome editing using CRISPR-Cas enzymes offer great therapeutic potential but off-target genome edits represent a safety concern. Direct intracellular delivery of ribonucleoprotein (RNP) genome editing complexes are preferable over the use of DNA delivery because of the speed of genome editing and rapid clearance of the RNP afterwards. Conventional methods rely on lipofection or electroporation for RNP delivery, which have their limitations for therapeutic uses. RNP conjugation to cell-penetrating peptides have also been explored with limited success. Improved technologies for intracellular delivery of RNPs are thus highly desirable.

SUMMARY

Synthetic peptide shuttle agents represent a recently defined family of peptides previously reported to transduce proteinaceous cargoes quickly and efficiently to the cytosol and/or nucleus of a wide variety of target eukaryotic cells. In contrast to traditional cell penetrating peptide-based intracellular delivery strategies, synthetic peptide shuttle agents are preferably not covalently linked to their polypeptide cargoes at the moment of delivery across the plasma membrane. In fact, covalently linking shuttle agents to their cargoes in a non-cleavable manner generally has a negative effect on their transduction activity. The first generation of such peptide shuttle agents was described in WO/2016/161516, wherein the peptide shuttle agents comprise an endosome leakage domain (ELD) operably linked to a cell penetrating domain (CPD). WO/2018/068135 subsequently described further synthetic peptide shuttle agents rationally-designed based on a set of fifteen design parameters for the sole purpose of improving the rapid transduction of proteinaceous cargoes, while reducing toxicity of the first generation peptide shuttle agents.

The majority of first and second generation shuttle agents are peptides at least twenty amino acids in length. Shuttle agent truncation experiments were undertaken herein to identify minimal fragments of first- and second-generation synthetic peptide shuttle agents sufficient for cargo transduction activity. These experiments revealed that C-terminal truncations were generally more tolerated than N-terminal truncations, with C-terminal truncations often retaining substantial cargo transduction activity when the N-terminal fragment is predicted to adopt a “core” region corresponding to an amphipathic cationic alpha helical structure when in solution at physiological conditions (e.g., at neutral pH). Common physiochemical properties of this core region and/or sub-20 amino acid shuttle agents are described herein.

In one aspect, described herein are synthetic peptide shuttle agents less than 20 amino acids in length having cargo transduction activity and their use for delivering a variety of cargoes in eukaryotic cells. The shuttle agents generally comprise a helical region comprising an amphipathic helix harboring: a cluster of hydrophobic amino acid residues on one side of the helix defining a hydrophobic angle of 140° to 280° in Schiffer-Edmundson’s wheel representation, and a cluster of positively charged residues on the other side of the helix defining a positively charged angle of 40° to 160° in Schiffer-Edmundson’s wheel representation.

While first- and second-generation shuttle agents efficiently deliver Cpf1-RNP (Cas12a-RNP) genome editing complexes to the nucleus of eukaryotic cells, they are shown herein to be less efficient at delivering Cas9-RNPs. While sharing similar sizes (SpCas9, 170 kDa and AsCpf1, 156 kDa), a major difference between the two enzymes likely influencing delivery is the net negative charge density contributed by their respective guide RNAs. AsCpf1 uses a simple crRNA (CRISPR RNA) (~42 nucleotides), and SpCas9 requires a crRNA and a tracrRNA (trans-activating crRNA) (~100 nucleotides). Described herein are synthetic peptide shuttle agents suitable for improved delivery of Cas-RNPs, which include shorter peptides, as well as peptides having reduced cationic charge density in one or both flanking segments.

In further aspects, described herein is a composition comprising a nucleoprotein cargo for intracellular delivery and a synthetic peptide shuttle agent independent from or not covalently linked to said nucleoprotein cargo, the synthetic peptide shuttle agent being a peptide comprising an amphipathic alpha-helical motif having both a positively-charged hydrophilic outer face and a hydrophobic outer face, wherein synthetic peptide shuttle agent increases cytosolic/nuclear delivery of said nucleoprotein cargo in eukaryotic cells as compared to in the absence of the synthetic peptide shuttle agent. In embodiments, the nucleoprotein cargo is a deoxyribonucleoprotein (DNP) or ribonucleoprotein (RNP) complex such as Cas9-RNP.

In some embodiments, the shuttle agents described herein may comprise a fragment of a parent shuttle agent as defined herein, wherein the fragment retains cargo transduction activity and comprises an amphipathic alpha-helical motif having both a positively-charged hydrophilic outer face and a hydrophobic outer face. In some embodiments, the shuttle agents described herein may comprise a variant of a parent shuttle agent as defined herein, wherein the variant retains cargo transduction activity and differs (or differs only) from the shuttle agent by having a reduced C-terminal positive charge density relative to the parent shuttle agent (e.g., by substituting one or more cationic residues, such as K/R, with non-cationic residues, preferably non-cationic hydrophilic residues). In some embodiments, the shuttle agent fragments and/or variants described herein have increased resistance to inhibition by nucleoproteins and/or extracellular DNA/RNA, and/or have increased transduction activity for nucleoprotein cargoes.

General Definitions

Headings, and other identifiers, e.g., (a), (b), (i), (ii), etc., are presented merely for ease of reading the specification and claims. The use of headings or other identifiers in the specification or claims does not necessarily require the steps or elements be performed in alphabetical or numerical order or the order in which they are presented.

The use of the word “**a**” or “**an**” when used in conjunction with the term “comprising” in the claims and/or the specification may mean “one” but it is also consistent with the meaning of “one or more”, “at least one”, and “one or more than one”.

The term “**about**”, when used herein, indicates that a value includes the standard deviation of error for the device or method being employed in order to determine the value. In general, the terminology “about” is meant to designate a possible variation of up to 10%. Therefore, a variation of 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10% of a value is included in the term “about”. Unless indicated otherwise, use of the term “about” before a range applies to both ends of the range.

As used in this specification and claim(s), the words “**comprising**” (and any form of comprising, such as “comprise” and “comprises”), “**having**” (and any form of having, such as “have” and “has”), “**including**” (and any form of including, such as “includes” and “include”) or “**containing**” (and any form of containing, such as “contains” and “contain”) are inclusive or open-ended and do not exclude additional, unrecited elements or method steps.

As used herein, “**protein**” or “**polypeptide**” or “**peptide**” means any peptide-linked chain of amino acids, which may or may not comprise any type of modification (e.g., chemical or post-translational modifications such as acetylation, phosphorylation, glycosylation, sulfatation, sumoylation, prenylation, ubiquitination, etc.). For further clarity, protein/polypeptide/peptide modifications are envisaged so long as the modification does not destroy the cargo transduction activity of the shuttle agents

described herein. For example, shuttle agents described herein may be linear or circular, may be synthesized with one or more D- or L-amino acids, and/or may be conjugated to a fatty acid (e.g., at their N terminus). Shuttle agents described herein may also have at least one amino acid being replaced with a corresponding synthetic amino acid having a side chain of similar physiochemical properties (e.g., structure, hydrophobicity, or charge) as the amino acid being replaced.

As used herein, a “**domain**” or “**protein domain**” generally refers to a part of a protein having a particular functionality or function. Some domains conserve their function when separated from the rest of the protein, and thus can be used in a modular fashion. The modular characteristic of many protein domains can provide flexibility in terms of their placement within the shuttle agents of the present description. However, some domains may perform better when engineered at certain positions of the shuttle agent (e.g., at the N- or C-terminal region, or therebetween). The position of the domain within its endogenous protein is sometimes an indicator of where the domain should be engineered within the shuttle agent and of what type/length of linker should be used. Standard recombinant DNA techniques can be used by the skilled person to manipulate the placement and/or number of the domains within the shuttle agents of the present description in view of the present disclosure. Furthermore, assays disclosed herein, as well as others known in the art, can be used to assess the functionality of each of the domains within the context of the shuttle agents (e.g., their ability to facilitate cell penetration across the plasma membrane, endosome escape, and/or access to the cytosol). Standard methods can also be used to assess whether the domains of the shuttle agent affect the activity of the cargo to be delivered intracellularly. In this regard, the expression “**operably linked**” as used herein refers to the ability of the domains to carry out their intended function(s) (e.g., cell penetration, endosome escape, and/or subcellular targeting) within the context of the shuttle agents of the present description. For greater clarity, the expression “operably linked” is meant to define a functional connection between two or more domains without being limited to a particular order or distance between same.

As used herein, the term “**synthetic**” used in expressions such as “synthetic peptide”, synthetic peptide shuttle agent”, or “synthetic polypeptide” is intended to refer to non-naturally occurring molecules that can be produced *in vitro* (e.g., synthesized chemically and/or produced using recombinant DNA technology). The purities of various synthetic preparations may be assessed by, for example, high-performance liquid chromatography analysis and mass spectroscopy. Chemical synthesis approaches may be advantageous over cellular expression systems (e.g., yeast or bacteria protein expression systems), as they may preclude the need for extensive recombinant protein purification steps (e.g., required for clinical use). In contrast, longer synthetic polypeptides may be more complicated and/or costly to produce via chemical synthesis approaches and such polypeptides may be more advantageously produced using cellular expression systems. In some embodiments, the peptides or shuttle agents of the present description may be chemically synthesized (e.g., solid- or liquid phase peptide synthesis), as opposed to expressed from a recombinant host cell. In some

embodiments, the peptides or shuttle agent of the present description may lack an N-terminal methionine residue. A person of skill in the art may adapt a synthetic peptide or shuttle agent of the present description by using one or more modified amino acids (e.g., non-naturally-occurring amino acids), or by chemically modifying the synthetic peptide or shuttle agent of the present description, to suit particular needs of stability or other needs.

As used herein, the term “**independent**” is generally intended refer to molecules or agents which are not covalently bound to one another, or that may be transiently covalently linked via a cleavable bond such that the molecules or agents (e.g., shuttle agent and cargo) detach from one another through cleavage of the bond following administration (e.g., when exposed to the reducing cellular environment, and/or but prior to, simultaneously with, or shortly after being delivered intracellularly). For example, the expression “independent cargo” is intended to refer to a cargo to be delivered intracellularly (transduced) that is not covalently bound (e.g., not fused) to a shuttle agent of the present description at the time of transduction across the plasma membrane. In some aspects, having shuttle agents that are independent of (not fused to) a cargo may be advantageous by providing increased shuttle agent versatility – e.g., being able to readily vary the ratio of shuttle agent to cargo (as opposed to being limited to a fixed ratio in the case of a covalent linkage between the shuttle agent and cargo). In some aspects, covalently linking a shuttle agent to its cargo via a cleavable bond such that they detach from one another upon contact with target cells may be advantageous from a manufacturing and/or regulatory perspective.

As used herein, the expression “**is or is from**” or “**is from**” comprises functional variants of a given protein or peptide (e.g., a shuttle agent described herein) or domain thereof (e.g., CPD or ELD), such as conservative amino acid substitutions, deletions, modifications, as well as variants or function derivatives, which do not abrogate the activity of the protein domain.

Other objects, advantages and features of the present description will become more apparent upon reading of the following non-restrictive description of specific embodiments thereof, given by way of example only with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

In the appended drawings:

Fig. 1 shows cargo transduction activity of short/truncated synthetic shuttle agents in HeLa cells for a small molecule cargo, propidium iodide (PI), and a proteinaceous cargo, GFP. The rows are ranked based on “Overall Delivery Factor”, a single calculated number that accounts for the toxicity of each shuttle agent/peptide, as well as its ability to deliver GFP and PI. Structural properties are shown for each peptide, including amino acid sequence, length, hydrophobic moment (μH), helical wheel projection, as

well as positively charged and hydrophobic angles. Results are means calculated from experiments performed at least in duplicate.

Fig. 2A shows the inhibitory effect of increasing amounts of sgRNA spiked in the transduction medium on shuttle agent-mediated transduction of a fluorescently-labeled cargo in RH-30 cells evaluated by flow cytometry. The shuttle agent was FSD250 and the cargo was an FITC-labelled phosphorodiamidate morpholino oligomer (PMO-FITC). **Fig. 2B** shows the results of a transduction assay in which HeLa cells are co-incubated with the shuttle agent FSD250 and GFP as a cargo either in the presence (+) or absence (-) of Cas9-RNP complex, at increasing concentrations of the small positively charged molecule 1,3-diaminoguanidine monohydrochloride as an RNA charge-neutralizing agent.

Results are means calculated from experiments performed at least in duplicate.

Fig. 3 shows the results of a transduction assays in which HeLa cells are co-incubated with different peptides/shuttle agents and GFP cargo, in the presence (+) or absence (-) of Cas9-RNP. Results are means calculated from experiments performed at least in duplicate.

Fig. 4A shows the change in GFP transduction efficiency in HeLa cells in the presence (+) or absence (-) of Cas9-RNP for the structurally-related peptides FSD10-15, FSD375, FSD422, FSD424, FSD432, FSD241, FSD231, FSD10, and FSD210.

Fig. 4B shows the change in GFP transduction efficiency in HeLa cells in the presence (+) or absence (-) of Cas9-RNP for the structurally-related peptides CM18, FSD440, CM18-L2-PTD4, His-CM18-Transportan, CM18-TAT, His-CM18-9Arg, and His-CM18-TAT.

Fig. 4C shows the change in GFP transduction efficiency in HeLa cells in the presence (+) or absence (-) of Cas9-RNP for the structurally-related peptides FSD356, FSD357, FSD446, FSD250, FSD296, FSD246, and FSD251.

Fig. 4D shows the change in GFP transduction efficiency in HeLa cells in the presence (+) or absence (-) of Cas9-RNP for the structurally-related peptides FSD374, FSD183, FSD168, FSD172, FSD189, FSD174, and FSD187.

Fig. 5 shows the change in GFP transduction efficiency in CFF-16HBEge cells in the presence (+) or absence (-) of Cas9-RNP for the structurally-related peptides FSD10 and FSD375. **Fig. 6A to Fig. 6E** each show the ability of structurally different shuttle agents to deliver functional Cpf1-RNP or Cas9-RNP genome editing complexes and effect genome editing in HeLa cells.

Fig. 7 shows the ability of structurally different shuttle agents to deliver functional Cpf1-RNP or Cas9-RNP genome editing complexes and effect genome editing in refractory Human Bronchial Epithelial cell line, CFF-16HBEge.

Fig. 8A to Fig. 8C compare the ability of the shuttle agent FSD10, and variants thereof, to deliver functional Cas9-RNP genome editing complexes versus ABE-Cas9-RNP base editing complexes in CFF-

16HBEge cells. **Fig. 9** shows the results of a large-scale screening of over 300 candidate peptide shuttle agents for PI and GFP-NLS transduction activity.

Fig. 10 shows core and side view 3D images of the peptides/shuttle agents of **Fig. 1** generated by PyMOL. Varying shades of green represent hydrophobic residues (Y, W, I, M, L, F), with darker green representing highly hydrophobic residues; blue residues represent charged hydrophilic residues (K, H, R, E, D); red residues represent uncharged hydrophilic residues (Q, N); and yellow/orange residues represent weakly hydrophobic residues (G, A, S, T).

SEQUENCE LISTING

This application contains a Sequence Listing in computer readable form created October 21, 2021. The computer readable form is incorporated herein by reference.

SEQ ID NO:	Description	29	FSD162	61	FSD121
1	CM18-Penetratin-cys	30	FSD168	62	FSD122
2	TAT-KALA	31	FSD173	63	FSD123
3	His-CM18-PTD4	32	FSD174	64	FSD124
4	His-LAH4-PTD4	33	FSD194	65	FSD125
5	PTD4-KALA	34	FSD220	66	FSD126
6	EB1-PTD4	35	FSD250	67	FSD127
7	His-CM18-PTD4-6Cys	36	FSD250D	68	FSD128
8	CM18-PTD4	37	FSD253	69	FSD130
9	CM18-PTD4-6His	38	FSD258	70	FSD132
10	His-CM18-PTD4-His	39	FSD262	71	FSD133
11	TAT-CM18	40	FSD263	72	FSD135
12	FSD5	41	FSD264	73	FSD137
13	FSD10	42	FSD265	74	FSD138
14	FSD12	43	FSD268	75	FSD139
15	FSD18	44	FSD286	76	FSD140
16	FSD19	45	FSD271	77	FSD141
17	FSD21	46	FSD272	78	FSD142
18	FSD23	47	FSD273	79	FSD143
19	FSD120	48	FSD276	80	FSD144
20	FSD127	49	FSD268 Cyclic Amide	81	FSD145
21	FSD129	50	FSD268 Cyclic Disulfide	82	FSD147
22	FSD131	51	FSD10 Scramble	83	FSD148
23	FSD134	52	FSD268 Scramble	84	FSD149
24	FSD146	53	FSD174 Scramble	85	FSD150
25	FSD155	54	FSN3	86	FSD151
26	FSD156	55	FSN4	87	FSD152
27	FSD157	56	FSN7	88	FSD153
28	FSD159	57	FSN8	89	FSD154
		58	FSD117	90	FSD158
		59	FSD118	91	FSD160
		60	FSD119	92	FSD161
				93	FSD163
				94	FSD164

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171	FSD248
172	FSD250 Scramble
173	FSD250E
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341	FSD434
342	FSD435
343	FSD436
344	FSD438
345	His-PTD4
346	FSD92
347	C(LLKK) ₃ C
348	CM18
349	FSD10-8
350	FSD10-12-1
351	FSD10-12-2
352	FSD10-15
353	FSD418-8
354	FSD418-12-1
355	FSD418-12-2
356	FSD418-15
357	FSD418-19
358	FSD439
359	FSD440

360	FSD441
361	FSD442
362	FSD443
363	KALA
364	FSD444
365	LAH4
366	Penetratin
367	PTD4
368	TAT
369	FSD445
370	FSD446
371	FSD447
372	crRNA beta-2 microglobulin (B2M) for Cas9
373	crRNA beta-2 microglobulin (B2M) for Cpf1
374	CM18-L2-PTD4
375	His-CM18- Transportan
376	CM18-TAT
377	His-CM18-9Arg
378	His-CM18-TAT
379	FSD448
380	Linker-(FSD10- Cter)-Linker
381	FSD10-Cter

DETAILED DESCRIPTION

In one aspect, described herein are short synthetic peptide shuttle agents having cargo transduction activity and their use for delivering a variety of independent cargoes in eukaryotic cells. As used herein, the expression “**short synthetic peptide shuttle agents**” or “**short shuttle agents**” may refer to synthetic peptide shuttle agents less than 20 amino acids in length or may refer to a “core” amphipathic cationic alpha helical region less than 20 amino acids in length within a longer shuttle agent.

In some embodiments, the short shuttle agents generally comprise a helical region comprising an amphipathic helix harboring: a cluster of hydrophobic amino acid residues on one side of the helix defining a hydrophobic angle of 140° to 280° in Schiffer-Edmundson’s wheel representation, and a cluster of positively charged residues on the other side of the helix defining a positively charged angle of 40° to 160° in Schiffer-Edmundson’s wheel representation. In some embodiments, the cluster of hydrophobic amino acid residues on one side of the helix define a hydrophobic angle of 140° to 280°, 160° to 260°, or 180° to 240° in Schiffer-Edmundson’s wheel representation. In some embodiments, the cluster of positively charged residues on the other side of the helix define a positively charged angle of 40° to 160°, 40° to 140°, or 60° to 140° in Schiffer-Edmundson’s wheel representation. The foregoing geometries were generally commonly shared by short shuttle agents, as described in **Example 3**.

In some embodiments, at least 20%, 30%, 40%, or 50% of the residues in the hydrophobic cluster are hydrophobic residues. In some embodiments, the hydrophobic residues are selected from the group consisting of phenylalanine, isoleucine, tryptophan, leucine, valine, methionine, tyrosine, cysteine, glycine, and alanine. In some embodiments, the hydrophobic residues are selected from the group consisting of phenylalanine, isoleucine, tryptophan, and/or leucine.

In some embodiments, at least 20%, 30%, 40%, or 50% of the residues in the positively charged cluster are positively charged residues. In some embodiments, the positively charged residues are selected from the group consisting of lysine, arginine, and histidine. In some embodiments, the positively charged residues are selected from the group consisting of lysine and arginine.

In some embodiments, the short synthetic peptide shuttle agent is at least 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, or 19 amino acids in length.

In some embodiments, the short synthetic peptide shuttle agent may have a hydrophobic moment (μH) of at least 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, 5.1, 5.2, 5.3, 5.4, or 5.5. Preferably, the short shuttle agents have a hydrophobic moment of at least 3.5, 4, or 4.5.

In some embodiments, the short shuttle agents may be used for transducing cargoes such as polypeptides, peptides, nucleoproteins, small molecules, or oligonucleotide analogs (e.g., non-anionic oligonucleotide analogs).

In some aspects, described herein are compositions and methods for nucleoprotein cargo transduction. The compositions generally comprise a nucleoprotein cargo for intracellular delivery and a synthetic peptide shuttle agent that is independent from, or is not covalently linked to, said nucleoprotein cargo. The synthetic peptide shuttle agent is a peptide comprising an amphipathic alpha-helical motif having both a positively-charged hydrophilic outer face and a hydrophobic outer face, wherein synthetic peptide shuttle agent increases cytosolic/nuclear delivery of said nucleoprotein cargo in eukaryotic cells as compared to in the absence of the synthetic peptide shuttle agent.

In some embodiments, the nucleoprotein cargo may be a deoxyribonucleoprotein (DNP) and/or ribonucleoprotein (RNP) complex. In some embodiments, the nucleoprotein cargo may be an RNA-guided nuclease, a Cas nuclease, such as a Cas type I, II, III, IV, V, or VI nuclease, or a variant thereof that lacking nuclease activity, a base editor, a CRISPR-associated transposase, a Cas-recombinase (e.g., recCas9), or a Cas prime editor. In some embodiments, the nucleoprotein cargo may be Cpf1-RNP (Cas12a-RNP) or Cas9-RNP. In some embodiments, the nucleoprotein cargo comprises a polynucleotide from 10 to 50 bases, 50 to 75 bases, 50 to 100 bases, 50 to 150 bases, 50 to 200 bases, 50 to 250 bases, 75 to 150 bases, or 75 to 125 bases.

In some embodiments, the nucleoprotein cargo is not covalently linked or pre-complexed with a cell-penetrating or cationic peptide. In some embodiments, the nucleoprotein cargo is not encapsulated or combined with a lipid-based carrier.

Rational design parameters and peptide shuttle agents

In some aspects, the shuttle agents described herein may be a peptide having transduction activity for nucleoprotein cargoes, proteinaceous cargoes, small molecules, non-anionic polynucleotide analogs, or any combination thereof, in target eukaryotic cells (WO/2018/068135, CA 3,040,645, WO/2020/210916, PCT/CA2021/051458).

In some embodiments, the shuttle agents described herein preferably satisfy one or more or any combination of the following fifteen rational design parameters.

(1) In some embodiments, the shuttle agent is a peptide at least 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acids in length. For example, the peptide may comprise a minimum length of 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 amino acid residues, and a maximum length of 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, or 150 amino acid residues. In some embodiments, shorter peptides (e.g., in the 17-50 or 20-50

amino acid range) may be particularly advantageous because they may be more easily synthesized and purified by chemical synthesis approaches, which may be more suitable for clinical use (as opposed to recombinant proteins that must be purified from cellular expression systems). While numbers and ranges in the present description are often listed as multiples of 5, the present description should not be so limited. For example, the maximum length described herein should be understood as also encompassing a length of 56, 57, 58...61, 62, etc., in the present description, and that their non-listing herein is only for the sake of brevity. The same reasoning applies to the % of identities listed herein.

(2) In some embodiments, the peptide shuttle agent comprises an amphipathic alpha-helical motif at neutral pH. As used herein, the expression “**alpha-helical motif**” or “**alpha-helix**”, unless otherwise specified, refers to a right-handed coiled or spiral conformation (helix) having angle of rotation between consecutive amino acids of 100 degrees and/or an alpha-helix having 3.6 residues per turn. As used herein, the expression “**comprises an alpha-helical motif**” or “**an amphipathic alpha-helical motif**” and the like, refers to the three-dimensional conformation that a peptide (or segment of a peptide) of the present description is predicted to adopt when in a biological setting at neutral pH based on the peptide’s primary amino acid sequence, regardless of whether the peptide actually adopts that conformation when used in cells as a shuttle agent. Furthermore, the peptides of the present description may comprise one or more alpha-helical motifs in different locations of the peptide. For example, the shuttle agent FSD5 in WO/2018/068135 is predicted to adopt an alpha-helix over the entirety of its length (see Figure 49C of WO/2018/068135), while the shuttle agent FSD18 of WO/2018/068135 is predicted to comprise two separate alpha-helices towards the N and C terminal regions of the peptide (see Figure 49D of WO/2018/068135). In some embodiments, the shuttle agents of the present description are not predicted to comprise a beta-sheet motif, for example as shown in Figures 49E and 49F of WO/2018/068135. Methods of predicting the presence of alpha-helices and beta-sheets in proteins and peptides are well known in the art. For example, one such method is based on 3D modeling using PEP-FOLD™, an online resource for de novo peptide structure prediction (<http://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD/>) (Lamiable et al., 2016; Shen et al., 2014; Thévenet et al., 2012). Other methods of predicting the presence of alpha-helices in peptides and protein are known and readily available to the skilled person.

As used herein, the expression “**amphipathic**” refers to a peptide that possesses both hydrophobic and hydrophilic elements (e.g., based on the side chains of the amino acids that comprise the peptide). For example, the expression “**amphipathic alpha helix**” or “**amphipathic alpha-helical motif**” refers to a peptide predicted to adopt an alpha-helical motif having a non-polar hydrophobic face and a polar hydrophilic face, based on the properties of the side chains of the amino acids that form the helix.

(3) In some embodiments, peptide shuttle agents of the present description comprise an amphipathic alpha-helical motif having a positively-charged hydrophilic outer face, such as one that is rich in R and/or K

residues. As used herein, the expression “**positively-charged hydrophilic outer face**” refers to the presence of at least three lysine (K) and/or arginine (R) residues clustered to one side of the amphipathic alpha-helical motif, based on alpha-helical wheel projection (e.g., see Figure 49A, left panel of WO/2018/068135). Such helical wheel projections may be prepared using a variety of programs, such as the online helical wheel projection tool created by Don Armstrong and Raphael Zidovetzki. (e.g., available at: <https://www.donarmstrong.com/cgi-bin/wheel.pl>) or the online tool developed by Mól et al., 2018 (e.g., available at <http://lbqp.unb.br/NetWheels/>). In some embodiments, the amphipathic alpha-helical motif may comprise a positively-charged hydrophilic outer face that comprises: (a) at least two, three, or four adjacent positively-charged K and/or R residues upon helical wheel projection; and/or (b) a segment of six adjacent residues comprising three to five K and/or R residues upon helical wheel projection, based on an alpha helix having angle of rotation between consecutive amino acids of 100 degrees and/or an alpha-helix having 3.6 residues per turn.

In some embodiments, peptide shuttle agents of the present description comprise an amphipathic alpha-helical motif comprising a hydrophobic outer face, the hydrophobic outer face comprising: (a) at least two adjacent L residues upon helical wheel projection; and/or (b) a segment of ten adjacent residues comprising at least five hydrophobic residues selected from: L, I, F, V, W, and M, upon helical wheel projection, based on an alpha helix having angle of rotation between consecutive amino acids of 100 degrees and/or an alpha-helix having 3.6 residues per turn.

(4) In some embodiments, peptide shuttle agents of the present description comprise an amphipathic alpha-helical motif having a highly hydrophobic core composed of spatially adjacent highly hydrophobic residues (e.g., L, I, F, V, W, and/or M). In some embodiments, the highly hydrophobic core may consist of spatially adjacent L, I, F, V, W, and/or M amino acids representing 12 to 50% of the amino acids of the peptide, calculated while excluding any histidine-rich domains (see below), based on an open cylindrical representation of the alpha-helix having 3.6 residues per turn, as shown for example in Figure 49A, right panel of WO/2018/068135. In some embodiments, the highly hydrophobic core may consist of spatially adjacent L, I, F, V, W, and/or M amino acids representing from 12.5%, 13%, 13.5%, 14%, 14.5%, 15%, 15.5%, 16%, 16.5%, 17%, 17.5%, 18%, 18.5%, 19%, 19.5%, or 20%, to 25%, 30%, 35%, 40%, or 45% of the amino acids of the peptide. More particularly, highly hydrophobic core parameter may be calculated by first arranging the amino acids of the peptide in an opened cylindrical representation, and then delineating an area of contiguous highly hydrophobic residues (L, I, F, V, W, M), as shown in Figure 49A, right panel of WO/2018/068135. The number of highly hydrophobic residues comprised in this delineated highly hydrophobic core is then divided by the total amino acid length of the peptide, excluding any histidine-rich domains (e.g., N- and/or C-terminal histidine-rich domains). For example, for the peptide shown in Figure 49A of WO/2018/068135,

there are 8 residues in the delineated highly hydrophobic core, and 25 total residues in the peptide (excluding the terminal 12 histidines). Thus, the highly hydrophobic core is 32% (8/25).

(5) **Hydrophobic moment** relates to a measure of the amphiphilicity of a helix, peptide, or part thereof, calculated from the vector sum of the hydrophobicities of the side chains of the amino acids (Eisenberg et al., 1982). An online tool for calculating the hydrophobic moment of a polypeptide is available from: <http://r2lab.ucr.edu/scripts/wheel/wheel.cgi>. A high hydrophobic moment indicates strong amphiphilicity, while a low hydrophobic moment indicates poor amphiphilicity. In some embodiments, peptide shuttle agents of the present description may consist of or comprise a peptide or alpha-helical domain having a hydrophobic moment (μ) of 3.5 to 11. In some embodiments, the shuttle agent may be a peptide comprising an amphipathic alpha-helical motif having a hydrophobic moment between a lower limit of 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7.0, and an upper limit of 9.5, 9.6, 9.7, 9.8, 9.9, 10.0, 10.1, 10.2, 10.3, 10.4, 10.5, 10.6, 10.7, 10.8, 10.9, or 11.0. In some embodiments, the shuttle agent may be a peptide having a hydrophobic moment between a lower limit of 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7.0, and an upper limit of 9.5, 9.6, 9.7, 9.8, 9.9, 10.0, 10.1, 10.2, 10.3, 10.4, or 10.5. In some embodiments, the hydrophobic moment is calculated excluding any histidine-rich domains that may be present in the peptide.

(6) In some embodiments, peptide shuttle agents of the present description may have a predicted net charge of at least +3 or +4 at physiological pH, calculated from the side chains of K, R, D, and E residues. For example, the net charge of the peptide may be at least +5, +6, +7, at least +8, at least +9, at least +10, at least +11, at least +12, at least +13, at least +14, or at least +15 at physiological pH. These positive charges are generally conferred by the greater presence of positively-charged lysine and/or arginine residues, as opposed to negatively charged aspartate and/or glutamate residues.

(7) In some embodiments, peptide shuttle agents of the present description may have a predicted isoelectric point (pI) of 8 to 13, preferably from 10 to 13. Programs and methods for calculating and/or measuring the isoelectric point of a peptide or protein are known in the art. For example, pI may be calculated using the Prot Param software available at: <http://web.expasy.org/protparam/>

(8) In some embodiments, peptide shuttle agents of the present description may be composed of 35 to 65% of hydrophobic residues (A, C, G, I, L, M, F, P, W, Y, V). In particular embodiments, the peptide shuttle agents may be composed of 36% to 64%, 37% to 63%, 38% to 62%, 39% to 61%, or 40% to 60% of any combination of the amino acids: A, C, G, I, L, M, F, P, W, Y, and V.

(9) In some embodiments, peptide shuttle agents of the present description may be composed of 0 to 30% of neutral hydrophilic residues (N, Q, S, T). In particular embodiments, the peptide shuttle agents may be

composed of 1% to 29%, 2% to 28%, 3% to 27%, 4% to 26%, 5% to 25%, 6% to 24%, 7% to 23%, 8% to 22%, 9% to 21%, or 10% to 20% of any combination of the amino acids: N, Q, S, and T.

(10) In some embodiments, peptide shuttle agents of the present description may be composed of 35 to 85% of the amino acids A, L, K and/or R. In particular embodiments, the peptide shuttle agents may be composed of 36% to 80%, 37% to 75%, 38% to 70%, 39% to 65%, or 40% to 60% of any combination of the amino acids: A, L, K, or R.

(11) In some embodiments, peptide shuttle agents of the present description may be composed of 15 to 45% of the amino acids A and/or L, provided there being at least 5% of L in the peptide. In particular embodiments, the peptide shuttle agents may be composed of 15% to 40%, 20% to 40%, 20 to 35%, or 20 to 30% of any combination of the amino acids: A and L, provided there being at least 5% of L in the peptide.

(12) In some embodiments, peptide shuttle agents of the present description may be composed of 20 to 45% of the amino acids K and/or R. In particular embodiments, the peptide shuttle agents may be composed of 20% to 40%, 20 to 35%, or 20 to 30% of any combination of the amino acids: K and R.

(13) In some embodiments, peptide shuttle agents of the present description may be composed of 0 to 10% of the amino acids D and/or E. In particular embodiments, the peptide shuttle agents may be composed of 5 to 10% of any combination of the amino acids: D and E.

(14) In some embodiments, the absolute difference between the percentage of A and/or L and the percentage of K and/or R in the peptide shuttle agent may be less than or equal to 10%. In particular embodiments, the absolute difference between the percentage of A and/or L and the percentage of K and/or R in the peptide shuttle agent may be less than or equal to 9%, 8%, 7%, 6%, or 5%.

(15) In some embodiments, peptide shuttle agents of the present description may be composed of 10% to 45% of the amino acids Q, Y, W, P, I, S, G, V, F, E, D, C, M, N, T, or H (i.e., not A, L, K, or R). In particular embodiments, the peptide shuttle agents may be composed of 15 to 40%, 20% to 35%, or 20% to 30% of any combination of the amino acids: Q, Y, W, P, I, S, G, V, F, E, D, C, M, N, T, and H.

In some embodiments, peptide shuttle agents of the present description respect at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, at least ten, at least eleven, at least twelve, at least thirteen, at least fourteen, or all of parameters (1) to (15) described herein. In particular embodiments, peptide shuttle agents of the present description respect all of parameters (1) to (3), and at least six, at least seven, at least eight, at least nine, at least ten, at least eleven, or all of parameters (4) to (15) described herein.

In some embodiments, where a peptide shuttle agent of the present description comprises only one histidine-rich domain, the residues of the one histidine-rich domain may be included in the calculation/assessment of parameters (1) to (15) described herein. In some embodiments, where a peptide shuttle agent of the present description comprises more than one histidine-rich domain, only the residues of

one of the histidine-rich domains may be included in the calculation/assessment of parameters (1) to (15) described herein. For example, where a peptide shuttle agent of the present description comprises two histidine-rich domains: a first histidine-rich domain towards the N terminus, and a second histidine-rich domain towards the C terminus, only the first histidine-rich domain may be included in the calculation/assessment of parameters (1) to (15) described herein.

In some embodiments, a machine-learning or computer-assisted design approach may be implemented to generate peptides that respect one or more of parameters (1) to (15) described herein. Some parameters, such as parameters (1) and (5)-(15), may be more amenable to implementation in a computer-assisted design approach, while structural parameters, such as parameters (2), (3) and (4), may be more amenable to a manual design approach. Thus, in some embodiments, peptides that respect one or more of parameters (1) to (15) may be generated by combining computer-assisted and manual design approaches. For example, multiple sequence alignment analyses of a plurality of peptides shown herein (and others) to function as effective shuttle agents revealed the presence of some consensus sequences – i.e., commonly found patterns of alternance of hydrophobic, cationic, hydrophilic, alanine and glycine amino acids. The presence of these consensus sequences are likely to give rise to structural parameters (2), (3) and (4) being respected (i.e., amphipathic alpha-helix formation, a positively-charged face, and a highly hydrophobic core of 12%-50%). Thus, these and other consensus sequences may be employed in machine-learning and/or computer-assisted design approaches to generate peptides that respect one or of parameters (1)-(15).

Accordingly, in some embodiments, peptide shuttle agents described herein may comprise or consist of the amino acid sequence of:

- (a) [X1]-[X2]-[linker]-[X3]-[X4] (Formula 1);
- (b) [X1]-[X2]-[linker]-[X4]-[X3] (Formula 2);
- (c) [X2]-[X1]-[linker]-[X3]-[X4] (Formula 3);
- (d) [X2]-[X1]-[linker]-[X4]-[X3] (Formula 4);
- (e) [X3]-[X4]-[linker]-[X1]-[X2] (Formula 5);
- (f) [X3]-[X4]-[linker]-[X2]-[X1] (Formula 6);
- (g) [X4]-[X3]-[linker]-[X1]-[X2] (Formula 7);
- (h) [X4]-[X3]-[linker]-[X2]-[X1] (Formula 8);
- (i) [linker]-[X1]-[X2]-[linker] (Formula 9);
- (j) [linker]-[X2]-[X1]-[linker] (Formula 10);
- (k) [X1]-[X2]-[linker] (Formula 11);
- (l) [X2]-[X1]-[linker] (Formula 12);
- (m) [linker]-[X1]-[X2] (Formula 13);
- (n) [linker]-[X2]-[X1] (Formula 14);

(o) [X1]-[X2] (Formula 15); or

(p) [X2]-[X1] (Formula 16),

wherein:

- [X1] is selected from: 2[Φ]-1[+]-2[Φ]-1[ζ]-1[+]-; 2[Φ]-1[+]-2[Φ]-2[+]-; 1[+]-1[Φ]-1[+]-2[Φ]-1[ζ]-1[+]-; and 1[+]-1[Φ]-1[+]-2[Φ]-2[+]-;
- [X2] is selected from: -2[Φ]-1[+]-2[Φ]-2[ζ]-; -2[Φ]-1[+]-2[Φ]-2[+]-; -2[Φ]-1[+]-2[Φ]-1[+]-1[ζ]-; -2[Φ]-1[+]-2[Φ]-1[ζ]-1[+]-; -2[Φ]-2[+]-1[Φ]-2[+]-; -2[Φ]-2[+]-1[Φ]-2[ζ]-; -2[Φ]-2[+]-1[Φ]-1[+]-1[ζ]-; and -2[Φ]-2[+]-1[Φ]-1[ζ]-1[+]-;
- [X3] is selected from: -4[+]-A-; -3[+]-G-A-; -3[+]-A-A-; -2[+]-1[Φ]-1[+]-A-; -2[+]-1[Φ]-G-A-; -2[+]-1[Φ]-A-A-; or -2[+]-A-1[+]-A-; -2[+]-A-G-A-; -2[+]-A-A-A-; -1[Φ]-3[+]-A-; -1[Φ]-2[+]-G-A-; -1[Φ]-2[+]-A-A-; -1[Φ]-1[+]-1[Φ]-1[+]-A-; -1[Φ]-1[+]-1[Φ]-G-A-; -1[Φ]-1[+]-1[Φ]-A-A-; -1[Φ]-1[+]-A-1[+]-A-; -1[Φ]-1[+]-A-G-A-; -1[Φ]-1[+]-A-A-A-; -A-1[+]-A-1[+]-A-; -A-1[+]-A-G-A-; and -A-1[+]-A-A-A-;
- [X4] is selected from: -1[ζ]-2A-1[+]-A-; -1[ζ]-2A-2[+]-; -1[+]-2A-1[+]-A-; -1[ζ]-2A-1[+]-1[ζ]-A-1[+]-; -1[ζ]-A-1[ζ]-A-1[+]-; -2[+]-A-2[+]-; -2[+]-A-1[+]-A-; -2[+]-A-1[+]-1[ζ]-A-1[+]-; -2[+]-1[ζ]-A-1[+]-; -1[+]-1[ζ]-A-1[+]-A-; -1[+]-1[ζ]-A-2[+]-; -1[+]-1[ζ]-A-1[+]-1[ζ]-A-1[+]-; -1[+]-2[ζ]-A-1[+]-; -1[+]-2[ζ]-2[+]-; -1[+]-2[ζ]-1[+]-A-; -1[+]-2[ζ]-1[+]-1[ζ]-A-1[+]-; -1[+]-2[ζ]-1[ζ]-A-1[+]-; -3[ζ]-2[+]-; -3[ζ]-1[+]-A-; -3[ζ]-1[+]-1[ζ]-A-1[+]-; -1[ζ]-2A-1[+]-A-; -1[ζ]-2A-2[+]-; -1[ζ]-2A-1[+]-1[ζ]-A-1[+]-; -2[+]-A-1[+]-A-; -2[+]-1[ζ]-1[+]-A-; -1[+]-1[ζ]-A-1[+]-A-; -1[+]-2A-1[+]-1[ζ]-A-1[+]-; and -1[ζ]-A-1[ζ]-A-1[+]-; and
- [linker] is selected from: -Gn-; -Sn-; -(GnSn)n-; -(GnSn)nGn-; -(GnSn)nSn-; -(GnSn)nGn(GnSn)n-; and -(GnSn)nSn(GnSn)n-;

wherein: [Φ] is an amino acid which is: Leu, Phe, Trp, Ile, Met, Tyr, or Val, preferably Leu, Phe, Trp, or Ile; [+] is an amino acid which is: Lys or Arg; [ζ] is an amino acid which is: Gln, Asn, Thr, or Ser; A is the amino acid Ala; G is the amino acid Gly; S is the amino acid Ser; and n is an integer from 1 to 20, 1 to 19, 1 to 18, 1 to 17, 1 to 16, 1 to 15, 1 to 14, 1 to 13, 1 to 12, 1 to 11, 1 to 10, 1 to 9, 1 to 8, 1 to 7, 1 to 6, 1 to 5, 1 to 4, or 1 to 3.

In some embodiments, peptide shuttle agents of the present description may comprise or consist of a peptide which is at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of any one of SEQ ID NOs: 1 to 50, 58 to 78, 80 to 107, 109 to 139, 141 to 146, 149 to 161, 163 to 169, 171, 174 to 234, 236 to 240, 242 to 260, 262 to 285, 287 to 294, 296 to 300, 302 to 308, 310, 311, 313 to 324, 326 to 332, 338 to 342, 344, 346, 348, 352, 355, 356, 358

to 360, 362, 363, 366, 369, 370, or 379 or to the amino acid sequence of any one of SEQ ID NOs: 104, 105, 107, 108, 110-131, 133-135, 138, 140, 142, 145, 148, 151, 152, 169-242, and 243-10 242 as disclosed in WO/2018/068135, or a functional variant thereof. In some embodiments, peptide shuttle agents of the present description may comprise the amino acid sequence motifs of SEQ ID NOs: 158 and/or 159 of

5 WO/2018/068135, which were found in each of peptides FSD5, FSD16, FSD18, FSD19, FSD20, FSD22, and FSD23. In some embodiments, peptide shuttle agents of the present description may comprise the amino acid sequence motif of SEQ ID NO: 158 of WO/2018/068135 operably linked to the amino acid sequence motif of SEQ ID NO: 159 of WO/2018/068135. As used herein, a “**functional variant**” refers to a peptide having cargo transduction activity, which differs from the reference peptide by one or more conservative amino acid
10 substitutions. As used herein in the context of functional variants, a “**conservative amino acid substitution**” is one in which one amino acid residue is replaced with another amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been well defined in the art, including basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side
15 chains (e.g., alanine, valine, leucine, isoleucine, phenylalanine, methionine, tryptophan, and optionally proline), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine).

In some embodiments, peptide shuttle agents of the present description do not comprise one or more of the amino acid sequences of any one of SEQ ID NOs: 57-59, 66-72, or 82-102 of WO/2018/068135. In
20 some embodiments, peptide shuttle agents of the present description do not comprise one or more of the amino acid sequences of any one of SEQ ID NOs: 104, 105, 107, 108, 110-131, 133-135, 138, 140, 142, 145, 148, 151, 152, 169-242, and 243-10 242 as disclosed in WO/2018/068135. Rather, in some embodiments, peptide shuttle agents of the present description may relate to variants of such previously described shuttle agent peptides, wherein the variants are further engineered for improved transduction activity (i.e., capable of more
25 robustly transducing nucleoprotein cargoes).

In some embodiments, peptide shuttle agents of the present description may have a minimal threshold of transduction efficiency and/or cargo delivery score for a “surrogate” cargo as measured in a eukaryotic cell model system (e.g., an immortalized eukaryotic cell line) or in a model organism. The expression
“**transduction efficiency**” refers to the percentage or proportion of a population of target cells into which a
30 cargo of interest is delivered intracellularly, which can be determined for example by flow cytometry, immunofluorescence microscopy, and other suitable methods may be used to assess cargo transduction efficiency (e.g., as described in WO/2018/068135). In some embodiments, transduction efficiency may be expressed as a percentage of cargo-positive cells. In some embodiments, transduction efficiency may be expressed as a fold-increase (or fold-decrease) over a suitable negative control assessed under identical

conditions except for in the absence of cargo and shuttle agent (“no treatment”; NT) or in the absence of shuttle agent (“cargo alone”).

In some embodiments, the shuttle agents described herein comprises or consists of:

- (i) the amino acid sequence any one of **SEQ ID NOs: 1 to 50, 58 to 78, 80 to 107, 109 to 139, 141 to 146, 149 to 161, 163 to 169, 171, 174 to 234, 236 to 240, 242 to 260, 262 to 285, 287 to 294, 296 to 300, 302 to 308, 310, 311, 313 to 324, 326 to 332, 338 to 342, 344, 346, 348, 352, 355, 356, 358 to 360, 362, 363, 366, 369, 370, or 379**;
- (ii) an amino acid sequence that differs from any one of **SEQ ID NOs: 1 to 50, 58 to 78, 80 to 107, 109 to 139, 141 to 146, 149 to 161, 163 to 169, 171, 174 to 234, 236 to 240, 242 to 260, 262 to 285, 287 to 294, 296 to 300, 302 to 308, 310, 311, 313 to 324, 326 to 332, 338 to 342, 344, 346, 348, 352, 355, 356, 358 to 360, 362, 363, 366, 369, 370, or 379** by no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acids (e.g., excluding any linker domains);
- (iii) an amino acid sequence that is at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to any one of **SEQ ID NOs: 1 to 50, 58 to 78, 80 to 107, 109 to 139, 141 to 146, 149 to 161, 163 to 169, 171, 174 to 234, 236 to 240, 242 to 260, 262 to 285, 287 to 294, 296 to 300, 302 to 308, 310, 311, 313 to 324, 326 to 332, 338 to 342, 344, 346, 348, 352, 355, 356, 358 to 360, 362, 363, 366, 369, 370, or 379** (e.g., calculated excluding any linker domains or glycine/serine-rich flanking domains);
- (iv) an amino acid sequence that differs from any one of **SEQ ID NOs: 1 to 50, 58 to 78, 80 to 107, 109 to 139, 141 to 146, 149 to 161, 163 to 169, 171, 174 to 234, 236 to 240, 242 to 260, 262 to 285, 287 to 294, 296 to 300, 302 to 308, 310, 311, 313 to 324, 326 to 332, 338 to 342, 344, , 346, 348, 352, 355, 356, 358 to 360, 362, 363, 366, 369, 370, or 379** by only conservative amino acid substitutions (e.g., by no more than no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 conservative amino acid substitutions, preferably excluding any linker domains or glycine/serine-rich flanking domains), wherein each conservative amino acid substitution is selected from an amino acid within the same amino acid class, the amino acid class being: Aliphatic: G, A, V, L, and I; Hydroxyl or sulfur/selenium-containing: S, C, U, T, and M; Aromatic: F, Y, and W; Basic: H, K, and R; Acidic and their amides: D, E, N, and Q; or
- (v) any combination of (i) to (iv).

In some embodiments, shuttle agents described herein are preferably second generation shuttle agents lacking a cell-penetrating domain or lack a cell-penetrating domain fused to an endosome leakage domain. In some embodiments, shuttle agents described herein particularly suitable for delivery of nucleoprotein cargoes

are preferably those having relatively high transduction efficiencies over high delivery scores, meaning that the shuttle agents deliver cargo to a greater percentage of cells (instead of a greater total number of cargo molecules per cell). Indeed, excess CRISPR-Cas genome editing complexes delivered intracellularly may increase the probability of off-target effects. In some embodiments, shuttle agents described herein (and/or the SEQ ID NOs recited above in the preceding paragraph) are those listed in Fig. 9 having a *Mean % of PI⁺ cells* or a *Mean % of GFP⁺ cells* of at least 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, or 75%.

In some embodiments, the shuttle agents described herein comprise or consist of a variant of the synthetic peptide shuttle agent, the variant being identical to the synthetic peptide shuttle agent as defined herein, except having at least one amino acid being replaced with a corresponding synthetic amino acid having a side chain of similar physiochemical properties (e.g., structure, hydrophobicity, or charge) as the amino acid being replaced, wherein the variant increases cytosolic/nuclear delivery of said cargo in eukaryotic cells as compared to in the absence of the synthetic peptide shuttle agent.

In some embodiments, the shuttle agents described herein may comprise or consist of a fragment of a longer parent shuttle agent as described or referred to herein, wherein the fragment retains cargo transduction activity and comprises an amphipathic alpha-helical motif having both a positively-charged hydrophilic outer face and a hydrophobic outer face. In some embodiments, the shuttle agents described herein may comprise or consist of a variant of a parent shuttle agent as described or referred to herein, wherein the variant retains cargo transduction activity and differs (or differs only) from the parent shuttle agent by having a reduced N-terminal and/or C-terminal positive charge density relative to the parent shuttle agent. As used herein “**positive charge density**” refers to the total number of residues with positively charged sidechains at physiological pH per length of the peptide. For example, three consecutive arginine residues (RRR) have a greater charge density than three arginine residues spaced farther apart by non-cationic residues (e.g., RARAR). In some embodiments, positive charge density may be reduced by substituting one or more cationic residues, such as K/R, with non-cationic residues, preferably non-cationic hydrophilic residues; and/or by engineering hydrophobic residues (e.g., A, V, L, I, F, or W) between two proximal cationic residues. In some embodiments, positive charge density may be reduced by increasing the distance between positive charge residues in close proximity in the peptide. In some embodiments, the shuttle peptide fragments or variants described herein, or the short shuttle agents described herein, preferably have increased resistance to inhibition by the nucleoprotein cargo, and/or has increased transduction activity for the nucleoprotein cargo. In some embodiments, shuttle peptide fragments or variants described herein, or the short shuttle agents described herein may comprise or consist of a C-terminal truncation of a longer parent shuttle agent.

In some embodiments, shuttle peptide fragments or variants described herein, or the short shuttle agents described herein, may comprise a “core” amphipathic alpha-helical motif having both a positively-

charged hydrophilic outer face and a hydrophobic outer face, which is flanked by or at least by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 non-cationic hydrophilic residues, such that the fragment or variant retains cargo transduction activity and/or has increased resistance to inhibition by the nucleoprotein cargo or by the presence of extracellular DNA/RNA.

Chemical modifications and synthetic amino acids

In some embodiments, shuttle agents of the present description may comprise oligomers (e.g., dimers, trimers, etc.) of peptides described herein. Such oligomers may be constructed by covalently binding the same or different types of shuttle agent monomers (e.g., using disulfide bridges to link cysteine residues introduced into the monomer sequences). In some embodiments, shuttle agents of the present description may comprise an N-terminal and/or a C-terminal cysteine residue.

In some embodiments, shuttle agents of the present description may comprise or consist of a cyclic peptide. In some embodiments, the cyclic peptide may be formed via a covalent link between a first residue positioned towards the N terminus of the shuttle agent and a second residue positioned towards the C terminus of the shuttle agent. In some embodiments, the first and second residues are flanking residues positioned at the N and the C termini of the shuttle agent. In some embodiments, the first and second residues may be linked via an amide linkage to form the cyclic peptide. In some embodiments, the cyclic peptide may be formed by a disulfide bond between two cysteine residues within the shuttle agent, wherein the two cysteine residues are positioned towards the N and C termini of the shuttle agent. In some embodiments, the shuttle agent may comprise, or be engineered to comprise, flanking cysteine residues at the N and C termini, which are linked via a disulfide bond to form the cyclic peptide. In some embodiments, the cyclic shuttle agents described herein may be more resistant to degradation (e.g., by proteases) and/or may have a longer half-life than a corresponding linear peptide.

In some embodiments, the shuttle agents of the present description may comprise one or more D-amino acids. In some embodiments, the shuttle agents of the present description may comprise a D-amino acid at the N and/or C terminus of the shuttle agent. In some embodiments, the shuttle agents may be comprised entirely of D-amino acids. In some embodiments, the shuttle agents described herein having one or more D-amino acids may be more resistant to degradation (e.g., by proteases) and/or may have a longer half-life than a corresponding peptide comprised of only L-amino acids.

In some embodiments, the shuttle agents of the present description may comprise a chemical modification to one or more amino acids, wherein the chemical modification does not destroy the transduction activity of the synthetic peptide shuttle agent. As used herein in this context, the term “**destroy**” means that the chemical modification irreversibly abolishes the cargo transduction activity of a

peptide shuttle agent described herein. Chemical modifications that may transiently inhibit, attenuate, or delay the cargo transduction activity of a peptide shuttle agent described herein may be included in the chemical modifications to the shuttle agents of the present description. In some embodiments, the chemical modification to any one of the shuttle agents described herein may be at the N and/or C terminus of the shuttle agent. Examples of chemical modifications include the addition of an acetyl group (e.g., an N-terminal acetyl group), a cysteamide group (e.g., a C-terminal cysteamide group), or a fatty acid (e.g., C4-C16, C6-C14, C6-C12, C6-C8, or C8 fatty acid, preferably being N-terminal).

In some embodiments, the shuttle agents of the present description comprise shuttle agent variants having cargo transduction activity in target eukaryotic cells, the variants being identical to any shuttle agent of the present description, except having at least one amino acid being replaced with a corresponding synthetic amino acid or amino acid analog having a side chain of similar physiochemical properties (e.g., structure, hydrophobicity, or charge) as the amino acid being replaced. In some embodiments, the synthetic amino acid replacement:

- (a) replaces a basic amino acids with any one of: α -aminoglycine, α,γ -diaminobutyric acid, ornithine, α,β -diaminopropionic acid, 2,6-diamino-4-hexynoic acid, β -(1-piperazinyl)-alanine, 4,5-dehydrolysine, δ -hydroxylysine, ω,ω -dimethylarginine, homoarginine, ω,ω' -dimethylarginine, ω -methylarginine, β -(2-quinolyl)-alanine, 4-aminopiperidine-4-carboxylic acid, α -methylhistidine, 2,5-diiodohistidine, 1-methylhistidine, 3-methylhistidine, spinacine, 4-aminophenylalanine, 3-aminotyrosine, β -(2-pyridyl)-alanine, or β -(3-pyridyl)-alanine;
- (b) replaces a non-polar (hydrophobic) amino acid with any one of: dehydro-alanine, β -fluoroalanine, β -chloroalanine, β -iodoalanine, α -aminobutyric acid, α -aminoisobutyric acid, β -cyclopropylalanine, azetidine-2-carboxylic acid, α -allylglycine, propargylglycine, tert-butylalanine, β -(2-thiazolyl)-alanine, thiaproline, 3,4-dehydropyrolidine, tert-butylglycine, β -cyclopentylalanine, β -cyclohexylalanine, α -methylproline, norvaline, α -methylvaline, penicillamine, β , β -dicyclohexylalanine, 4-fluoroproline, 1-aminocyclopentanecarboxylic acid, pipercolic acid, 4,5-dehydroleucine, allo-isoleucine, norleucine, α -methylleucine, cyclohexylglycine, cis-octahydroindole-2-carboxylic acid, β -(2-thienyl)-alanine, phenylglycine, α -methylphenylalanine, homophenylalanine, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid, β -(3-benzothienyl)-alanine, 4-nitrophenylalanine, 4-bromophenylalanine, 4-tert-butylphenylalanine, α -methyltryptophan, β -(2-naphthyl)-alanine, β -(1-naphthyl)-alanine, 4-iodophenylalanine, 3-fluorophenylalanine, 4-fluorophenylalanine, 4-methyltryptophan, 4-chlorophenylalanine, 3,4-dichloro-phenylalanine, 2,6-difluoro-phenylalanine, n-in-methyltryptophan, 1,2,3,4-tetrahydronorharman-3-carboxylic acid, β,β -diphenylalanine, 4-methylphenylalanine, 4-phenylphenylalanine, 2,3,4,5,6-pentafluoro-phenylalanine, or 4-benzoylphenylalanine;

- (c) replaces a polar, uncharged amino acid with any one of: β -cyanoalanine, β -ureidoalanine, homocysteine, allo-threonine, pyroglutamic acid, 2-oxothiazolidine-4-carboxylic acid, citrulline, thiocitrulline, homocitrulline, hydroxyproline, 3,4-dihydroxyphenylalanine, β -(1,2,4-triazol-1-yl)-alanine, 2-mercaptohistidine, β -(3,4-dihydroxyphenyl)-serine, β -(2-thienyl)-serine, 4-azidophenylalanine, 4-cyanophenylalanine, 3-hydroxymethyltyrosine, 3-iodotyrosine, 3-nitrotyrosine, 3,5-dinitrotyrosine, 3,5-dibromotyrosine, 3,5-diiodotyrosine, 7-hydroxy-1,2,3,4-tetrahydroiso-quinoline-3-carboxylic acid, 5-hydroxytryptophan, thyronine, β -(7-methoxycoumarin-4-yl)-alanine, or 4-(7-hydroxy-4-coumarinyl)-aminobutyric acid; and/or
- (d) replaces an acidic amino acid with any one of: γ -hydroxyglutamic acid, γ -methyleneglutamic acid, γ -carboxyglutamic acid, α -aminoadipic acid, 2-aminoheptanedioic acid, α -aminosuberic acid, 4-carboxyphenylalanine, cysteic acid, 4-phosphonophenylalanine, or 4-sulfomethylphenylalanine.

Histidine-rich domains

In some embodiments, shuttle agents of the present description may further comprise one or more histidine-rich domains. In some embodiments, the histidine-rich domain may be a stretch of at least 2, at least 3, at least 4, at least 5, or at least 6 amino acids comprising at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, or at least 90% histidine residues. In some embodiments, the histidine-rich domain may comprise at least 2, at least 3, at least 4 at least 5, at least 6, at least 7, at least 8, or at least 9 consecutive histidine residues.

Without being bound by theory, in the context of first generation shuttle agents comprising a CPD operably linked to an ELD, the histidine-rich domain in the shuttle agent may act as a proton sponge in the endosome through protonation of their imidazole groups under acidic conditions of the endosomes, providing another mechanism of endosomal membrane destabilization and thus further facilitating the ability of endosomally-trapped cargoes to gain access to the cytosol. In some embodiments, the histidine-rich domain may be located at or towards the N and/or C terminus of the peptide shuttle agent.

Linkers

In some embodiments, peptide shuttle agents of the present description may comprise one or more **suitable linkers** (e.g., flexible polypeptide linkers). In some embodiments, such linkers may separate two or more amphipathic alpha-helical motifs (e.g., see the shuttle agent FSD18 in Figure 49D of WO/2018/068135), or a core amphipathic cationic motif from another motif. In some embodiments, linkers can be used to separate two more domains (CPDs, ELDs, or histidine-rich domains) from one another. In some embodiments, linkers may be formed by adding sequences of small hydrophobic amino acids with or without rotatory potential (such as glycine) and polar serine residues that confer stability and flexibility. Linkers may be soft and allow the

domains of the shuttle agents to move. In some embodiments, prolines may be avoided since they can add significant conformational rigidity. In some embodiments, the linkers may be serine/glycine-rich linkers. In some embodiments, the use shuttle agents comprising a suitable linker may be advantageous for delivering a cargo to suspension cells, rather than to adherent cells. In some embodiments, the linker may comprise or consist of: -Gn- ; -Sn- ; -(GnSn)_n- ; -(GnSn)_nGn- ; -(GnSn)_nSn- ; -(GnSn)_nGn(GnSn)_n- ; or -(GnSn)_nSn(GnSn)_n-, wherein G is the amino acid Gly; S is the amino acid Ser; and n is an integer from 1 to 5. In some embodiments, short stretches or “linkers” of flexible and/or hydrophilic amino acids (e.g., glycine/serine-rich stretches) may be added to the N terminus, C terminus, or both the N and C termini of a shuttle agent or core alpha helical amphipathic cationic domain described herein, or a C-terminal truncated shuttle agent described herein. In some embodiments, such stretches may facilitate dissolution of shuttle agents, particularly shorter shuttle agents (e.g., having an amphipathic alpha helical structure with a strongly hydrophobic portion) that would otherwise be insoluble or only partially soluble in aqueous solution. In some embodiments, increasing the solubility of shuttle agent peptides may avoid the use of organic solvents (e.g., DMSO) that may obscure cargo transduction results and/or make the shuttle agents incompatible for therapeutic applications. In some embodiments, the presence of flexible linkers flanking a central core alpha helical amphipathic cationic domain may provide enhanced resistance of the shuttle agent to inhibition by nucleoproteins and/or extracellular DNA/RNA.

Domain-based peptide shuttle agents

In some aspects, the shuttle agents described herein may be a first generation shuttle agent as described in WO/2016/161516, comprising an endosome leakage domain (ELD) operably linked to a cell penetrating domain (CPD).

Endosome leakage domains (ELDs)

In some aspects, peptide shuttle agents of the present description may comprise an endosome leakage domain (ELD) having endosomolytic activity. As used herein, the expression “**endosome leakage domain**” refers to a sequence of amino acids which confers the ability of endosomally-trapped cargoes to gain access to the cytoplasmic compartment. Without being bound by theory, endosome leakage domains are short sequences (often derived from viral or bacterial peptides), which are believed to induce destabilization of the endosomal membrane and liberation of the endosome contents into the cytoplasm. As used herein, the expression “**endosomolytic**” or “**endosomolytic peptide**” is intended to refer to this general class of peptides having endosomal membrane-destabilizing properties. Accordingly, in some embodiments, synthetic peptide or polypeptide-based shuttle agents of the present description may comprise an ELD which is an endosomolytic peptide. The activity of such peptides may be assessed for example using the calcein endosome escape assays described in Example 2 of WO/2016/161516.

In some embodiments, the ELD may be a peptide that disrupts membranes at acidic pH, such as pH-dependent membrane active peptide (PMAP) or a pH-dependent lytic peptide. For example, the peptides GALA and INF-7 are amphiphilic peptides that form alpha helices when a drop in pH modifies the charge of the amino acids which they contain. More particularly, without being bound by theory, it is suggested that ELDs such as GALA induce endosomal leakage by forming pores and flip-flop of membrane lipids following conformational change due to a decrease in pH (Kakudo et al., 2004; Li et al., 2004). In contrast, it is suggested that ELDs such as INF-7 induce endosomal leakage by accumulating in and destabilizing the endosomal membrane (El-Sayed et al., 2009). Accordingly, in the course of endosome maturation, the concomitant decline in pH causes a change in the conformation of the peptide and this destabilizes the endosome membrane leading to the liberation of the endosome contents. The same principle is thought to apply to the toxin A of *Pseudomonas* (Varkouhi et al., 2011). Following a decline in pH, the conformation of the domain of translocation of the toxin changes, allowing its insertion into the endosome membrane where it forms pores (London, 1992; O'Keefe, 1992). This eventually favors endosome destabilization and translocation of the complex outside of the endosome. The above described ELDs are encompassed within the ELDs of the present description, as well as other mechanisms of endosome leakage whose mechanisms of action may be less well defined.

In some embodiments, the ELD may be an antimicrobial peptide (AMP) such as a linear cationic alpha-helical antimicrobial peptide (AMP). These peptides play a key role in the innate immune response due to their ability to strongly interact with bacterial membranes. Without being bound by theory, these peptides are thought to assume a disordered state in aqueous solution, but adopt an alpha-helical secondary structure in hydrophobic environments. The latter conformation thought to contribute to their typical concentration-dependent membrane-disrupting properties. When accumulated in endosomes at certain concentrations, some antimicrobial peptides may induce endosomal leakage.

In some embodiments, the ELD may be an antimicrobial peptide (AMP) such as Cecropin-A/Melittin hybrid (CM) peptide. Such peptides are thought to be among the smallest and most effective AMP-derived peptides with membrane-disrupting ability. Cecropins are a family of antimicrobial peptides with membrane-perturbing abilities against both Gram-positive and Gram-negative bacteria. Cecropin A (CA), the first identified antibacterial peptide, is composed of 37 amino acids with a linear structure. Melittin (M), a peptide of 26 amino acids, is a cell membrane lytic factor found in bee venom. Cecropin-melittin hybrid peptides have been shown to produce short efficient antibiotic peptides without cytotoxicity for eukaryotic cells (i.e., non-hemolytic), a desirable property in any antibacterial agent. These chimeric peptides were constructed from various combinations of the hydrophilic N-terminal domain of Cecropin A with the hydrophobic N-terminal domain of Melittin, and have been tested on bacterial model systems. Two 26-mers, CA(1-13)M(1-13) and

CA(1-8) M(1-18) (Boman et al., 1989), have been shown to demonstrate a wider spectrum and improved potency of natural Cecropin A without the cytotoxic effects of melittin.

In an effort to produce shorter CM series peptides, the authors of Andreu et al., 1992 constructed hybrid peptides such as the 26-mer (CA(1-8)M(1-18)), and compared them with a 20-mer (CA(1-8)M(1-12)), a 18-mer (CA(1-8)M(1-10)) and six 15-mers ((CA(1-7)M(1-8), CA(1-7)M(2-9), CA(1-7)M(3-10), CA(1-7)M(4-11), CA(1-7)M(5-12), and CA(1-7)M(6-13)). The 20 and 18-mers maintained similar activity comparatively to CA(1-8)M(1-18). Among the six 15-mers, CA(1-7)M(1-8) showed low antibacterial activity, but the other five showed similar antibiotic potency compared to the 26-mer without hemolytic effect. Accordingly, in some embodiments, synthetic peptide or polypeptide-based shuttle agents of the present description may comprise an ELD which is or is from CM series peptide variants, such as those described above.

In some embodiments, the ELD may be the CM series peptide CM18 composed of residues 1–7 of Cecropin-A (KWKLFFKIGAVLKVLTTG) fused to residues 2–12 of Melittin (YGRKKRRQRRR), [C(1–7)M(2–12)]. When fused to the cell penetrating peptide TAT, CM18 was shown to independently cross the plasma membrane and destabilize the endosomal membrane, allowing some endosomally-trapped cargoes to be released to the cytosol (Salomone et al., 2012). However, the use of a CM18-TAT11 peptide fused to a fluorophore (atto-633) in some of the authors' experiments, raises uncertainty as to the contribution of the peptide versus the fluorophore, as the use of fluorophores themselves have been shown to contribute to endosomolysis -- e.g., via photochemical disruption of the endosomal membrane (Erazo-Oliveras et al., 2014).

In some embodiments, the ELD may be CM18 having the amino acid sequence of SEQ ID NO: 1 of WO/2016/161516, or a variant thereof having at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, or 95% identity to SEQ ID NO: 1 of WO/2016/161516 and having endosomolytic activity.

In some embodiments, the ELD may be a peptide derived from the N terminus of the HA2 subunit of influenza hemagglutinin (HA), which may also cause endosomal membrane destabilization when accumulated in the endosome.

In some embodiments, synthetic peptide or polypeptide-based shuttle agents of the present description may comprise an ELD which is or is from an ELD set forth in **Table I**, or a variant thereof having endosome escape activity and/or pH-dependent membrane disrupting activity.

Table I: Examples of endosome leakage domains

Name	SEQ ID NO of WO/2016/161516	Reference(s)
CM18	1	Salomone et al., 2012
Diphtheria toxin T domain (DT)	2	Uherek et al., 1998; Glover et al., 2009
GALA	3	Parente et al., 1990; Li et al., 2004

PEA	4	Fominaya and Wels 1996
INF-7	5	El-Sayed et al., 2009
LAH4	6	Kichler et al., 2006; Kichler et al., 2003
HGP	7	Kwon et al., 2010
H5WYG	8	Midoux et al., 1998
HA2	9	Lorieau et al., 2010
EB1	10	Amand et al., 2012
VSVG	11	Schuster et al., 1999
<i>Pseudomonas</i> toxin	12	Fominaya et al., 1998
Melittin	13	Tan et al., 2012
KALA	14	Wyman et al., 1997
JST-1	15	Gottschalk et al., 1996
C(LLKK) ₃ C	63	Luan et al., 2015
G(LLKK) ₃ G	64	Luan et al., 2015

In some embodiments, shuttle agents of the present description may comprise one or more ELD or type of ELD. More particularly, they can comprise at least 2, at least 3, at least 4, at least 5, or more ELDs. In some embodiments, the shuttle agents can comprise between 1 and 10 ELDs, between 1 and 9 ELDs, between 1 and 8 ELDs, between 1 and 7 ELDs, between 1 and 6 ELDs, between 1 and 5 ELDs, between 1 and 4 ELDs, between 1 and 3 ELDs, etc.

In some embodiments, the order or placement of the ELD relative to the other domains (CPD, histidine-rich domains) within the shuttle agents of the present description may be varied provided the shuttling ability of the shuttle agent is retained.

In some embodiments, the ELD may be a variant or fragment of any one those listed in **Table I**, and having endosomolytic activity. In some embodiments, the ELD may comprise or consist of the amino acid sequence of any one of SEQ ID NOs: 1-15, 63, or 64 of WO/2016/161516, or a sequence which is at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 85%, 90%, 91%, 92%, 93%, 94%, or 95% identical to any one of SEQ ID NOs: 1-15, 63, or 64 of WO/2016/161516, and having endosomolytic activity.

In some embodiments, shuttle agents of the present description do not comprise one or more of the amino acid sequences of any one of SEQ ID NOs: 1-15, 63, or 64 of WO/2016/161516.

Cell penetration domains (CPDs)

In some aspects, the shuttle agents of the present description may comprise a cell penetration domain (CPD). As used herein, the expression “**cell penetration domain**” refers to a sequence of amino acids which confers the ability of a macromolecule (e.g., peptide or protein) containing the CPD to be transduced into a cell.

In some embodiments, the CPD may be (or may be from) a cell-penetrating peptide or the protein transduction domain of a cell-penetrating peptide. Cell-penetrating peptides can serve as carriers to

successfully deliver a variety of cargoes intracellularly (e.g., polynucleotides, polypeptides, small molecule compounds or other macromolecules/compounds that are otherwise membrane-impermeable). Cell-penetrating peptides often include short peptides rich in basic amino acids that, once fused (or otherwise operably linked) to a macromolecule, mediate its internalization inside cells (Shaw et al., 2008). The first cell-penetrating peptide was identified by analyzing the cell penetration ability of the HIV-1 trans-activator of transcription (Tat) protein (Green and Loewenstein 1988, Vives et al., 1997). This protein contains a short hydrophilic amino acid sequence, named “TAT”, which promotes its insertion within the plasma membrane and the formation of pores. Since this discovery, many other cell-penetrating peptides have been described. In this regard, in some embodiments, the CPD can be a cell-penetrating peptide as listed in **Table II**, or a variant thereof having cell-penetrating activity.

Table II: Examples of cell-penetrating peptides

Name	SEQ ID NO of WO/2016/161516	Reference(s)
SP	16	Mahlum et al., 2007
TAT	17	Green and Loewenstein 1988; Fawell et al., 1994; Vives et al., 1997
Penetratin (Antennapedia)	18	Perez et al., 1992
pVEC	19	Elmqvist et al., 2001
M918	20	El-Andaloussi et al., 2007
Pep-1	21	Morris et al., 2001
Pep-2	22	Morris et al., 2004
Xentry	23	Montrose et al., 2013
Arginine stretch	24	Zhou et al., 2009
Transportan	25	Hallbrink et al., 2001
SynB1	26	Drin et al., 2003
SynB3	27	Drin et al., 2003
PTD4	65	Ho et al., 2001

Without being bound by theory, cell-penetrating peptides are thought to interact with the cell plasma membrane before crossing by pinocytosis or endocytosis. In the case of the TAT peptide, its hydrophilic nature and charge are thought to promote its insertion within the plasma membrane and the formation of a pore (Herc and Garcia, 2007). Alpha helix motifs within hydrophobic peptides (such as SP) are also thought to form pores within plasma membranes (Veatch et al., 2004).

In some embodiments, shuttle agents of the present description may comprise one or more CPD or type of CPD. More particularly, they may comprise at least 2, at least 3, at least 4, or at least 5 or more CPDs. In some embodiments, the shuttle agents can comprise between 1 and 10 CPDs, between 1 and 6 CPDs, between 1 and 5 CPDs, between 1 and 4 CPDs, between 1 and 3 CPDs, etc.

In some embodiments, the CPD may be TAT having the amino acid sequence of SEQ ID NO: 17 of WO/2016/161516, or a variant thereof having at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%,

79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, or 95% identity to SEQ ID NO: 17 of WO/2016/161516 and having cell penetrating activity; or Penetratin having the amino acid sequence of SEQ ID NO: 18 of WO/2016/161516, or a variant thereof having at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, or 95% identity to SEQ ID NO: 18 of WO/2016/161516 and having cell penetrating activity.

In some embodiments, the CPD may be PTD4 having the amino acid sequence of SEQ ID NO: 65 of WO/2016/161516, or a variant thereof having at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, or 95% identity to SEQ ID NO: 65 of WO/2016/161516.

In some embodiments, the order or placement of the CPD relative to the other domains (ELD, histidine-rich domains) within the shuttle agents of the present description may be varied provided the transduction ability of the shuttle agent is retained.

In some embodiments, the CPD may be a variant or fragment of any one those listed in **Table II**, and having cell penetrating activity. In some embodiments, the CPD may comprise or consist of the amino acid sequence of any one of SEQ ID NOs: 16-27 or 65 of WO/2016/161516, or a sequence which is at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 85%, 90%, 91%, 92%, 93%, 94%, or 95% identical to any one of SEQ ID NOs: 16-27 or 65 of WO/2016/161516, and having cell penetrating activity.

In some embodiments, shuttle agents of the present description do not comprise any one of the amino acid sequences of SEQ ID NOs: 16-27 or 65 of WO/2016/161516.

Methods, kits, uses, compositions, and cells

In some embodiments, the present description relates to methods for delivering cargoes from an extracellular space to the cytosol and/or nucleus of a target eukaryotic cell. The methods comprise contacting the target eukaryotic cell with the cargo in the presence of a shuttle agent at a concentration sufficient to increase the transduction efficiency of said cargo, as compared to in the absence of said shuttle agent. In some embodiments, contacting the target eukaryotic cell with the cargo in the presence of the shuttle agent results in an increase in the transduction efficiency of said cargo by at least 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 20, 30, 40, 50, or 100-fold, as compared to in the absence of said shuttle agent. In some embodiments, the concentration of cargo and/or of synthetic peptide shuttle agent in compositions described herein may be at least 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 μ M.

In some embodiments, the present description relates to a method for increasing the transduction efficiency of a cargo to the cytosol and/or nucleus of target eukaryotic cells. As used herein, the expression “**increasing transduction efficiency**” refers to the ability of a shuttle agent of the present description to improve the percentage or proportion of a population of target cells into which a cargo of interest is delivered intracellularly. Immunofluorescence microscopy, flow cytometry, and other suitable methods may be used to assess cargo transduction efficiency. In some embodiments, a shuttle agent of the present description may enable a transduction efficiency of at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, or 85%, for example as measured by immunofluorescence microscopy, flow cytometry, FACS, and other suitable methods. In some embodiments, a shuttle agent of the present description may enable one of the aforementioned transduction efficiencies together with a cell viability of at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95%, for example as measured by the assay described in Example 3.3a of WO/2018/068135, or by another suitable assay known in the art.

In addition to increasing target cell transduction efficiency, shuttle agents of the present description may facilitate the delivery of a cargo of interest to the cytosol and/or nucleus of target cells. In this regard, efficiently delivering an extracellular cargo to the cytosol and/or nucleus of a target cell using peptides can be challenging, as the cargo often becomes trapped in intracellular endosomes after crossing the plasma membrane, which may limit its intracellular availability and may result in its eventual metabolic degradation. For example, use of the protein transduction domain from the HIV-1 Tat protein has been reported to result in massive sequestration of the cargo into intracellular vesicles. In some aspects, shuttle agents of the present description may facilitate the ability of endosomally-trapped cargo to escape from the endosome and gain access to the cytoplasmic compartment. In this regard, the expression “**to the cytosol**” for example in the phrase “increasing the transduction efficiency of a cargo to the cytosol,” is intended to refer to the ability of shuttle agents of the present description to allow an intracellularly delivered cargo of interest to escape endosomal entrapment and gain access to the cytoplasmic and/or nuclear compartment. After a cargo of interest has gained access to the cytosol, it may be free to bind to its intracellular target (e.g., in the cytosol, nucleus, nucleolus, mitochondria, peroxisome). In some embodiments, the expression “to the cytosol” is thus intended to encompass not only cytosolic delivery, but also delivery to other subcellular compartments that first require the cargo to gain access to the cytoplasmic compartment.

In some embodiments, the methods of the present description are *in vitro* methods (e.g., such as for therapeutic and/or diagnostic purpose). In other embodiments, the methods of the present description are *in vivo* methods (e.g., such as for therapeutic and/or diagnostic purpose). In some embodiments, the methods of the present description comprise topical, enteral/gastrointestinal (e.g., oral), or parenteral administration of the cargo and the synthetic peptide shuttle agent. In some embodiments, described herein are compositions

formulated for topical, enteral/gastrointestinal (e.g., oral), or parenteral administration of the cargo and the synthetic peptide shuttle agent.

In some embodiments, the methods of the present description may comprise contacting the target eukaryotic cell with the shuttle agent, or composition as defined herein, and the cargo. In some embodiments, the shuttle agent, or composition may be pre-incubated with the cargo to form a mixture, prior to exposing the target eukaryotic cell to that mixture. In some embodiments, the type of shuttle agent may be selected based on the identity and/or physicochemical properties of the cargo to be delivered intracellularly. In other embodiments, the type of shuttle agent may be selected to take into account the identity and/or physicochemical properties of the cargo to be delivered intracellularly, the type of cell, the type of tissue, etc.

In some embodiments, the method may comprise multiple treatments of the target cells with the shuttle agent, or composition (e.g., 1, 2, 3, 4 or more times per day, and/or on a pre-determined schedule). In such cases, lower concentrations of the shuttle agent, or composition may be advisable (e.g., for reduced toxicity). In some embodiments, the cells may be suspension cells or adherent cells. In some embodiments, the person of skill in the art will be able to adapt the teachings of the present description using different combinations of shuttles, domains, uses and methods to suit particular needs of delivering a cargo to particular cells with a desired viability.

In some embodiments, the methods of the present description may apply to methods of delivering a cargo intracellularly to a cell *in vivo*. Such methods may be accomplished by parenteral administration or direct injection into a tissue, organ, or system.

In some aspects, the compositions or synthetic peptide shuttle agents of the present description may be for use in an *in vitro* or *in vivo* method for increasing the transduction efficiency of a cargo (e.g., a therapeutically or biologically relevant molecule or drug) into target eukaryotic cells, wherein the synthetic peptide shuttle agent or synthetic peptide shuttle agent variant is used or is formulated for use at a concentration sufficient to increase the transduction efficiency and cytosolic and/or nuclear delivery of the cargo into the target eukaryotic cells, as compared to in the absence of the synthetic peptide shuttle agent or synthetic peptide shuttle agent variant.

In some embodiments, compositions or synthetic peptide shuttle agents of the present description may be for use in therapy, wherein the synthetic peptide shuttle agent or synthetic peptide shuttle agent variant transduces a therapeutically relevant cargo to the cytosol and/or nucleus of target eukaryotic cells, wherein the synthetic peptide shuttle agent or synthetic peptide shuttle agent variant is used (or is formulated for use) at a concentration sufficient to increase the transduction efficiency of the cargo into the target eukaryotic cells, as compared to in the absence of the synthetic peptide shuttle agent.

In some aspects, described herein is a composition for use in transducing a cargo into target eukaryotic cells, the composition comprising a synthetic peptide shuttle agent formulated with a

pharmaceutically suitable excipient, wherein the concentration of the synthetic peptide shuttle agent in the composition is sufficient to increase the transduction efficiency and cytosolic and/or nuclear delivery of the cargo into said target eukaryotic cells upon administration, as compared to in the absence of said synthetic peptide shuttle agent. In some embodiments, the composition further comprises the cargo. In
5 some embodiments, the composition may be mixed with the cargo prior to administration or therapeutic use.

In some aspects, described herein is a composition for use in therapy, the composition comprising a synthetic peptide shuttle agent formulated with a cargo to be transduced into target eukaryotic cells by the synthetic peptide shuttle agent, wherein the concentration of the synthetic peptide shuttle agent in the
10 composition is sufficient to increase the transduction efficiency and cytosolic and/or nuclear delivery of the cargo into said target eukaryotic cells upon administration, as compared to in the absence of said synthetic peptide shuttle agent.

In some aspects, described herein is a composition: (a) for use in increasing the transduction efficiency of the nucleoprotein cargo to the cytosolic/nuclear compartment of eukaryotic cells; (b) for use
15 in genome editing, base editing, or prime editing in eukaryotic cells; (c) for use in modulating gene expression in the eukaryotic cells; (d) for use in therapy, wherein the nucleoprotein cargo binds to a therapeutic target in the eukaryotic cells; (e) for use in delivering a non-therapeutic nucleoprotein cargo as a diagnostic agent; (f) for use in the manufacture of a medicament or diagnostic agent; (g) for use in treating cancer (e.g., skin cancer, basal cell carcinoma, nevoid basal cell carcinoma syndrome),
20 inflammation or an inflammation-related disease (e.g., psoriasis, atopic dermatitis, ulcerative colitis, urticaria, dry eye disease, dry or wet age-related macular degeneration, digital ulcers, actinic keratosis, idiopathic pulmonary fibrosis), pain (e.g., chronic or acute), or a disease affecting the lungs (e.g., cystic fibrosis, asthma, chronic obstructive pulmonary disease (COPD), or idiopathic pulmonary fibrosis); or (h) any combination of (a) to (g).

In some aspects, described herein is a composition comprising a cargo for intracellular delivery and a synthetic peptide shuttle agent that is independent from, or is not covalently linked to, said cargo, the synthetic peptide shuttle agent being a peptide comprising an amphipathic alpha-helical motif having both a positively-charged hydrophilic outer face and a hydrophobic outer face, wherein synthetic peptide shuttle agent increases cytosolic/nuclear delivery of said cargo in eukaryotic cells as compared to in the
30 absence of the synthetic peptide shuttle agent. In some embodiments, the compositions and/or shuttle agents described herein do not comprise an organic solvent (e.g., DMSO), or do not comprise a concentration of an organic solvent not suitable for therapeutic or human use. In some embodiments, the shuttle agents described herein are advantageously designed with aqueous solubility in mind, thereby precluding the necessity of using organic solvents.

In some embodiments, the shuttle agent, or composition, and the cargo may be exposed to the target cell in the presence or absence of serum. In some embodiments, the method may be suitable for clinical or therapeutic use.

5 In some embodiments, the present description relates to a kit for delivering a cargo from an extracellular space to the cytosol and/or nucleus of a target eukaryotic cell. In some embodiments, the present description relates to a kit for increasing the transduction efficiency of a cargo to the cytosol of a target eukaryotic cell. The kit may comprise the shuttle agent, or composition as defined herein, and a suitable container.

10 In some embodiments, the target eukaryotic cells may be an animal cell, a mammalian cell, or a human cell. In some embodiments, the target eukaryotic cells may be stem cells (e.g., embryonic stem cells, pluripotent stem cells, induced pluripotent stem cells, neural stem cells, mesenchymal stem cells, hematopoietic stem cells, peripheral blood stem cells), primary cells (e.g., myoblast, fibroblast), immune cells (e.g., NK cell, T cell, dendritic cell, antigen presenting cell), epithelial cells, skin cells, gastrointestinal cells, mucosal cells, or pulmonary (lung) cells. In some embodiments, target cells comprise those having the
15 cellular machinery for endocytosis (i.e., to produce endosomes).

In some embodiments, the present description relates to an isolated cell comprising a synthetic peptide shuttle agent as defined herein. In some embodiments, the cell may be a pluripotent stem cell. It will be understood that cells that are often resistant or not amenable to DNA transfection may be interesting candidates for the synthetic peptide shuttle agents of the present description.

20 Synthetic peptide shuttle agents have been shown to enable efficient delivery of recombinant protein cargoes to refractory airway epithelial cells (Krishnamurthy et al., 2018). Mucus/sputum, particularly in subjects with respiratory diseases (e.g., cystic fibrosis), is known to be elevated in DNA (Chance et al., 2020), which may have an inhibitory effect on some synthetic peptide shuttle agents. In some aspects, described herein is a synthetic peptide shuttle agent for use in, or suitable for use in, the
25 delivery of non-anionic cargoes across mucus-producing membranes (e.g., airway epithelium), the synthetic peptide shuttle agent comprising or consisting essentially of a central core amphipathic alpha helical region having shuttle agent activity, flanked N- and C-terminally by flexible linker domains, wherein one or both of the flexible linker domains comprises or consists essentially of a sufficient number of non-cationic hydrophilic residues such that cargo transduction activity across mucus-producing
30 membranes of the synthetic peptide shuttle agent is increased relative to that of the central core amphipathic alpha helical region lacking the flexible linker domains. In some embodiments, the central core amphipathic alpha helical region: (a) may be an endosomolytic peptide; (b) may be at least 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, or 19 amino acids in length; (c) may be a fragment of a parent shuttle agent as defined in claim 14(a) or 15; (d) may be an amphipathic helix as defined in any one of claims 18 to 29

or 49 to 60; (e) may have a hydrophobic moment (μ_H) of at least 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, 5.1, 5.2, 5.3, 5.4, or 5.5; or (f) any combination of (a) to (e). In some embodiments, the non-cationic hydrophilic residues may comprise or consist essentially of glycine, serine, aspartate, glutamate, histidine, tyrosine, threonine, cysteine, asparagine, glutamine, or any combination thereof. In some embodiments, the flexible linker domain is any linker domain as defined herein.

EXAMPLES

Example 1: Materials and Methods

All materials and methods not described or specified herein were generally as performed in WO/2018/068135, CA 3,040,645, WO/2020/210916, or PCT/CA2021/051458. Materials and reagents used in the Examples herein are shown in **Table III**. All cell lines were grown according to the manufacture's instructions, as shown in **Table IV**.

15 **Helical Wheel Projections and generation of 3D peptide images**

Helical wheel projection images of the synthetic peptide shuttle agents in **Fig. 1** were generated using an online helical wheel projection tool created by Don Armstrong and Raphael Zidovetzki. (e.g., available at: <https://www.donarmstrong.com/cgi-bin/wheel.pl>). In **Fig. 10**, 3D peptide structures were built in PyMol (open-source version for Linux Version 2.4.0a0) using the "fab" command and the "ss=1" argument for the peptide to adopt an alpha helix conformation. Orientation was done manually. Colouring was done using a script, whereby varying shades of green represent strongly hydrophobic residues (Y, W, I, M, L, F), with darker green representing highly hydrophobic residues; blue residues represent charged hydrophilic residues (K, H, R, E, D); red residues represent uncharged hydrophilic residues (Q, N); and yellow/orange residues represent weakly hydrophobic residues (G, A, S, T).

25

Transduction protocol

Transduction of GFP-NLS

HeLa cells were plated (20 000 cells/well) in a 96 well-plate the day prior to the experiment in DMEM containing 10% FBS. Each delivery mix comprising a synthetic peptide shuttle agent (10-30 μ M) and GFP-NLS (10 μ M) was prepared and completed to 50 μ L with RPMI 1640 media. Cells were washed once with phosphate-buffered saline (PBS) and the shuttle/GFP-NLS mix was added to the cells and incubated for five minutes. Then 100 μ L DMEM containing 10% FBS was added to the mix. Cells were

30

then immediately washed once with PBS and incubated in DMEM containing 10% FBS for two hours. Cells were then analyzed by flow cytometry.

Transduction of PI

5 HeLa cells were plated (20 000 cells/well) in a 96 well-plate the day prior to the experiment in DMEM containing 10% FBS. Each delivery mix comprising a synthetic peptide shuttle agent (10-30 μ M) and the propidium iodide (PI) (10 μ g/mL) were prepared and completed to 50 μ L with PBS. Cells were washed once with PBS and the shuttle/PI mix was added to the cells and incubated for one minute. Then 100 μ L DMEM containing 10% FBS was added to the mix. Cells were then immediately washed once
10 with PBS and incubated in DMEM containing 10% FBS for two hours. Cells were then analyzed by flow cytometry.

Table III: Materials and Reagents

Materials	Company	City, Province-State, Country
DMEM	Sigma-Aldrich	Oakville, ON, Canada
Fetal bovine serum (FBS)	NorthBio	Toronto, ON, Canada
L-glutamine-Penicillin-Streptomycin	Sigma-Aldrich	Oakville, ON, Canada
RPMI 1640 media	Sigma-Aldrich	Oakville, ON, Canada
Trypsin-EDTA solution	Sigma-Aldrich	Oakville, ON, Canada
Propidium iodide	Sigma Aldrich/P4170-10MG	Oakville, ON, Canada
Alpha-MEM	Sigma-Aldrich	Oakville, ON, Canada
Edit-R TM TracrRNA	Horizon Discovery/ Dharmacon (U-002005-50)	Lafayette, CO, USA
crRNA Beta-2 microglobulin (B2M) for Cas9 GAGUAGCGCGAGCACAGCUA [SEQ ID NO: 372]	IDT	Mississauga, ON, Canada
crRNA Beta-2 microglobulin (B2M) for Cas12a AGUGGGGGUGAAUUCAGUGUAGU [SEQ ID NO: 373]	IDT	Mississauga, ON, Canada
1,3-Diaminoguanidine monohydrochloride	Sigma-Aldrich (#143413)	Oakville, ON, Canada
3,5-Diamino-1,2,4-triazole	Sigma-Aldrich (#D26202)	Oakville, ON, Canada
Guanidine hydrochloride	Sigma-Aldrich (#G3272)	Oakville, ON, Canada
L-Arginine amide dihydrochloride	Santa Cruz biotech. (sc-286061)	Dallas, TX, USA
Anti-beta 2 Microglobulin antibody – PE conjugated	Abcam (#ab49424)	Toronto, ON, Canada
Bovine Serum Albumin (BSA)	BioShop Canada Inc. #ALB007.100	Burlington, ON, Canada

15 **Table IV: Cell lines**

Cell lines	Description	ATCC/others	Culture media	Serum	Additives
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HeLa	Human cervical carcinoma cells	ATCC™ CCL-2	DMEM	10% FBS	L-glutamine 2 mM Penicillin 100 units Streptomycin 100µg/mL
CFF-16HBEge	Immortalized Human bronchial epithelial cells	Provided by the Cystic Fibrosis Foundation	Alpha-MEM	10% FBS	L-glutamine 2 mM Penicillin 100 units Streptomycin 100µg/mL
RH-30	Human rhabdomyosarcoma cell line	ATCC™ CRL-2061	RPMI-1640	10% FBS	L-glutamine 2 mM Penicillin 100 units Streptomycin 100µg/mL

GFP-NLS transduction in the presence of Cas9-RNP complex

HeLa cells were plated (20 000 cells/well) in a 96 well-plate the day prior to the experiment in DMEM containing 10% FBS. A mix was prepared containing a synthetic peptide shuttle agent (10-30 µM), the GFP-NLS (10 µM) with or without a Cas9-NLS recombinant protein (2.5 µM) complexed with a crRNA/tracrRNA (2µM) targeting the beta-2 microglobulin (B2M) gene and completed to 50 µL with PBS. Cells were washed once with PBS and the shuttle/GFP-NLS/Cas9-RNP mix was added to the cells and incubated for one minute. Then 100 µL DMEM containing 10% FBS was added to the mix. Cells were then immediately washed once with PBS and incubated in DMEM containing 10% FBS for two hours. Cells were the analyzed by flow cytometry.

GFP-NLS transduction in presence of Cas9-RNP complex coated with small molecules protocol

HeLa cells were plated (20 000 cells/well) in a 96 well-plate the day prior to the experiment in DMEM containing 10% FBS. The Cas9-RNP complex coated with small molecules was prepared by mixing a Cas9-NLS recombinant protein (5 µM) complexed with a crRNA/tracrRNA (4µM) targeting the beta-2 microglobulin (B2M) gene with 0, 100 nM, 1 µM, 10 µM, 100 µM, 1 mM or 10 mM of either 1,3-diaminoguanidine monohydrochloride, 3,5-diamino-1,2,4-triazole, guanidine hydrochloride or L-arginine amide dihydrochloride. The Cas9 RNP complex coated with small molecules was completed to 25µL with PBS. The delivery mix was prepared by mixing a synthetic peptide shuttle agent (10µM), the GFP-NLS (10 µM) with or without the Cas9 RNP complex coated or not with small molecules and completed to 50 µL with phosphate-buffered saline PBS. Cells were washed once with PBS and the shuttle/GFP-NLS/Cas9-RNP mix was added to the cells and incubated for one minute. Then 100 µL DMEM containing 10% FBS was added to the mix. Cells were then immediately washed once with PBS and incubated in DMEM containing 10% FBS for two hours. Cells were then analyzed by flow cytometry.

Transduction of CRISPR Cas9- or Cpf1-RNP

Transduction

HeLa cells were plated (10 000 cells/well) in a 96 well-plate the day prior to the experiment in DMEM containing 10% FBS. CFF-16HBEge cells were plated (10 000 cells/well) in a 96 well-plate the day prior to the experiment in Alpha-MEM containing 10% FBS.

For Cpf1-RNP transduction, a mix of Cpf1-NLS recombinant protein (1.33 μ M) complexed with a crRNA (2 μ M) targeting the beta-2 microglobulin (B2M) gene were co-incubated with 10-20 μ M of synthetic peptide shuttle agent in a final volume of 50 μ L completed with PBS. Cells were washed once with PBS and the shuttle/Cpf1-RNP mix was added to the cells and incubated for 90 seconds. Then 100 μ L of DMEM (HeLa) or Alpha-MEM (CFF-16HBEge) containing 10% FBS was added to the mix. Cells were then immediately washed once with PBS and incubated in DMEM (HeLa) or Alpha-MEM (CFF-16HBEge) containing 10% FBS.

For Cas9-RNP or ABE-Cas9-RNP transduction, a mix of Cas9-NLS or ABE-Cas9 recombinant protein (2.5 μ M) complexed with a crRNA/tracrRNA (2 μ M) targeting the beta-2 microglobulin (B2M) gene were co-incubated with 10-20 μ M of synthetic peptide shuttle agent in a final volume of 50 μ L completed with PBS. Cells were washed once with PBS and the shuttle/Cas9-RNP complex was added to the cells for and incubated for 60 to 90 seconds. Then 100 μ L of DMEM (HeLa) or Alpha-MEM (CFF-16HBEge) containing 10% FBS was added to the mix. Cells were then immediately washed once with PBS and incubated in DMEM (HeLa) or Alpha-MEM (CFF-16HBEge) containing 10% FBS.

Knockout analysis by flow cytometry

Genome editing events resulting in the absence of B2M protein (knockout) at the cell surface were determined by flow cytometry 6 days post-transduction. Cells were washed once with PBS and incubated with the anti-B2 microglobulin antibody (PE conjugated) (0.5 μ L of anti-B2M-PE in 50 μ L 0.5% BSA/PBS) for 45 minutes at room temperature. Cells were washed twice with PBS and detached with 50 μ L of Trypsin-EDTA for 10 minutes at 37°C then inactivated by adding 100 μ L of media containing 10% FBS. The percentage of knockout cells (cells without B2M antibody signal) was determined by flow cytometry.

For all transduction experiments, cell viabilities were above 75% unless otherwise indicated.

Example 2: Synthetic peptide shuttle agents: a new class of intracellular delivery peptides

Synthetic peptides called shuttle agents represent a new class of intracellular delivery peptides having the ability to rapidly transduce polypeptide cargoes to the cytosolic/nuclear compartment of eukaryotic cells. In contrast to traditional cell penetrating peptide-based intracellular delivery strategies, synthetic peptide shuttle agents are independent from, or are not covalently linked to, their polypeptide cargoes at the moment of transduction across the plasma membrane. In fact, covalently linking shuttle

agents to their cargoes in an uncleavable manner generally has a negative effect on their transduction activity.

The first generation of synthetic peptide shuttle agents was described in WO/2016/161516 and consisted of multi-domain-based peptides having an endosome leakage domain (ELD) operably linked to a cell penetrating domain (CPD), and optionally further comprising one or more histidine-rich domains. Although it was initially believed that shuttle agent-mediated cargo transduction occurred via mechanisms similar to that of conventional cell-penetrating peptides, the speed and efficiency of cargo delivery to the cytosolic/nuclear compartment suggested a strong contribution from a more direct delivery mechanism across the plasma membrane without requiring complete endosomal formation (Del'Guidice et al., 2018). Therefore, using the first-generation shuttle agents as a starting point, a large scale iterative design and screening program was undertaken to optimize the shuttle agents for the rapid and efficient transduction of polypeptide cargoes while reducing cellular toxicity. The program involved the manual and computer-assisted design/modeling of almost 11,000 synthetic peptides, as well as the synthesis and testing of several hundred different peptides for their ability to transduce a variety of polypeptide cargoes rapidly and efficiently in a plurality of cells and tissues. Rather than considering the shuttle agents as fusions of known cell-penetrating peptides (CPDs) and endosomolytic peptides (ELDs) derived from the literature, each peptide was considered holistically based on their predicted three-dimensional structure and physicochemical properties. The design and screening program culminated in a second generation of synthetic peptide shuttle agents defined by a set of fifteen parameters described in WO/2018/068135 governing the rational design of shuttle agents with improved transduction/toxicity profiles for polypeptide cargoes over the first generation shuttle agents. These second generation synthetic peptide shuttle agents were designed and empirically screened for the rapid transduction of polypeptide cargoes (i.e., typically within under 5 minutes) and thus were predominantly designed to lack a prototypical CPD.

Example 3: Truncated synthetic peptide shuttle agents retain transduction activity

Shuttle agent truncation experiments were undertaken to identify minimal fragments of first- and second-generation synthetic peptide shuttle agents sufficient for cargo transduction activity. These experiments revealed that C-terminal truncations were generally more tolerated than N-terminal truncations, with C-terminal truncations often retaining substantial cargo transduction activity when the N-terminal fragment was predicted to adopt an amphipathic cationic alpha helical structure when in solution at physiological conditions.

To test the transduction activity of short/truncated synthetic shuttle agents (e.g., generally having less than 20 amino acids), HeLa cells were incubated with control peptides and N-terminal shuttle agent fragments of different lengths and delivery of GFP or PI was assessed by flow cytometry as described in

Example 1. The results shown in **Fig. 1** rank the delivery of GFP and PI by each shuttle agent or controls (non-treated [NT] and GFP/PI only [no shuttle agent]) and are ranked according to their “Overall Delivery Factor”. The Overall Delivery Factor represents a single number that accounts for the toxicity of each shuttle agent/peptide, as well as its ability to deliver GFP and PI, and was calculated as follows:

$$\frac{(\text{mean PI delivery score}) \times (\text{mean viability [PI]}) + (\text{mean GFP delivery score}) \times (\text{mean viability [GFP]})}{2}$$

Shuttle agents having an Overall Delivery Factor greater than 0.5 possessed generally common characteristics (**Fig. 1**). Typically, these shuttle agents had a hydrophobic moment (μH) of at least 4. Furthermore, when projected into a Schiffer-Edmundson's wheel representation (helical wheel projection) depicting an amphipathic alpha-helical motif, the shuttle agents possessed hydrophobic and positively charged outer surfaces bearing particular angles and a certain percentage of specific residues. According to a typical Schiffer-Edmundson's wheel representation of 18 amino acids, the angle between two consecutive amino acids is 20 degrees, as described in Schiffer et al., 1967. Here, we determine the hydrophobic angle by first determining a region or cluster rich in hydrophobic amino acids and multiply 20 degrees by the number of spaces between each consecutive amino acid in the region or cluster. Similarly, the positively charged angle is calculated by first determining a region or cluster rich in the positively charged residues lysine (K) and arginine (R). The K and R residues most often consecutively appear, but the region or cluster may also comprise weakly or non-hydrophobic residues. In most cases, effective shuttle agents had a positively charged region defined by an angle between 60 and 120 degrees comprised of over 50% lysine (K) and/or arginine (R) residues. The larger hydrophobic angle of these shuttle agents were mostly defined between 180 and 240 degrees and comprised of over 50% phenylalanine (F), isoleucine (I), leucine (L) and/or tryptophan (W). Similar observations were made for shuttle agents longer than 20 amino acids comprising linker sequences or histidine-rich domains. Interestingly, transduction activity was observed for the CM18 peptide (18 amino acids long) in this experiment, which is an N-terminal fragment (endosome leakage domain) in some first generation shuttle agents. Computer-generated 3D images of the peptides of **Fig. 1** are shown in core and side views in **Fig. 9**.

Example 4: Inhibition of shuttle agent transduction activity by Cas9-RNP complexes not alleviated by coating with charge-neutralizing agents

Nuclear delivery of Cas9-sgRNA complexes (hereinafter Cas9-RNP) via first- and second-generation synthetic peptide shuttle agents generally occurs less efficiently than delivery of a Cas9 proteinaceous cargo alone (i.e., without its corresponding sgRNA; see Krishnamurthy et al., 2019,

supplementary Figure 6). The negative effect of the sgRNA (without Cas9) on shuttle agent-mediated transduction of a fluorescently-labelled charge-neutral polynucleotide analog cargo (phosphorodiamidate morpholino oligomer (PMO)) is also shown in **Fig. 2A**. Briefly, RH-30 cells (150,000 cells/well in 24-well dish) were contacted with a delivery mix containing 6 μ M of PMO-FITC and 5 μ M of the synthetic peptide shuttle agent FSD250 for 2 minutes in RPMI, in the presence of increasing amounts of sgRNA spiked in the medium. Cells were then washed, incubated in complete medium and then collected for analysis by flow cytometry after 1 h. The results in **Fig. 2A** show that reduced cargo transduction efficiency was observed in the presence of 2 μ g of sgRNA (4 μ g/mL).

Hypothesizing that the inhibitory effect of the sgRNA was due to the negatively charged phosphate backbone of the RNA, we attempted to neutralize the negative charges by coating the Cas9-RNP complexes with small positively charged molecules prior to transduction. Delivery of GFP in HeLa cells in the presence of Cas9-RNP was assessed in the presence of small positively charged molecules such as 1,3-diaminoguanidine monohydrochloride; 3,5-diamino-1,2,4-triazole; guanidine hydrochloride; or L-arginine amide dihydrochloride. As shown in **Fig. 2B**, the presence of Cas9-RNP significantly inhibited both GFP transduction activity ("Mean % cells GFP+") and Mean GFP delivery Scores, despite the presence of up to 10 mM of 1,3-diaminoguanidine monohydrochloride. Similar results were seen in the presence of 3,5-diamino-1,2,4-triazole; guanidine hydrochloride; or L-arginine amide dihydrochloride (data not shown).

Example 5: Shuttle agents having increased resistance to inhibition by Cas9-RNP

To better understand the inhibitory effect of Cas9-RNP on shuttle agent transduction activity, HeLa cells were incubated with different peptides/shuttle agents and GFP cargo, in the presence or absence of Cas9-RNP, and delivery of GFP was assessed by flow cytometry, as described in **Example 1**. As shown in **Fig. 3**, the presence of Cas9-RNP decreased the transduction efficiency of the GFP cargo for the majority of shuttle agents. For some shuttle agents, the effect was particularly striking. For example, the GFP transduction efficiency for FSD268 decreased from 92% to 29%, the GFP transduction efficiency for FSD250 decreased from 83% to 13%, the GFP transduction efficiency for FSD10 decreased from 76% to 22%, and the GFP transduction efficiency for FSD395 decreased from 91% to 17%. A subset of shuttle agents, however, showed a degree of resistance to the negative effects of Cas9-RNP. These more resistant peptides included FSD10-15, CM18, and FSD356. Structure-activity relationships were explored further by repeating the above transduction experiments with shuttle agent variants sharing the same "core" amphipathic cationic alpha helical region as FSD10-15 (**Fig. 4A**), CM18 (**Fig. 4B**), and FSD356 (**Fig. 4C**).

For FSD10-15, GFP transduction efficiency slightly *increased* from 21% to 24% in the presence of Cas9-RNP (**Fig. 3**). Interestingly, FSD10-15 is a 15-amino acid fragment of several longer shuttle agents, including FSD375, FSD422, FSD424, FSD432, FSD241, FSD231, FSD10, and FSD210. As shown in **Fig. 4A**, adding flanking glycine/serine-rich residues to FSD10-15 (see FSD375 and FSD424) retained the peptide's resistance to Cas9-RNP while improving GFP transduction activity over FSD10-15. Replacing the glycine/serine-rich residues with flanking histidine residues (see FSD422) did not maintain the same level of Cas9-RNP resistance. Of note, histidine-rich domains are capable of becoming increasingly cationic at pH values approaching the pKa of their imidazole side chains (about 6). Finally, the presence of a C-terminal glycine/serine-rich linker fused to a second cationic domain (FSD432, FSD241, FSD231, FSD10, and FSD210) seemed to render the shuttle agents more sensitive to inhibition by Cas9-RNP, with the effect FSD10 and FSD210 being particularly pronounced. FSD231 differs from FSD210 only by the insertion of a single leucine residue (L) immediately preceding the most C-terminal lysine residue (K), thereby decreasing the C-terminal positive charge density of FSD231 relative to FSD210. Interestingly, the insertion of this hydrophobic leucine residue rendered FSD231 substantially more resistant to inhibition by Cas9-RNP as compared to FSD210 (**Fig. 4A**).

For CM18, GFP transduction efficiency remained similar in the absence (32%) and presence (28%) of Cas9-sgRNA (**Fig. 3**). As shown in **Fig. 3** and **4B**, adding flanking glycine/serine-rich residues to CM18 (see FSD440) retained the peptide's resistance to Cas9-RNP but GFP delivery score was increased by two-fold (1.8 for CM18 to 3.6 for FSD440). Shuttle agents having high C-terminal positive charge densities (e.g., CM18-TAT, His-CM18-9Arg, and His-CM18-TAT) exhibited particularly marked sensitivities to inhibition by Cas9-RNP (**Fig. 4B**), while shuttle agents with lower C-terminal positive charge densities (e.g., CM18-L2-PTD4 and His-CM18-Transportan) were substantially more resistant to inhibition by Cas9-RNP (**Fig. 4B**). The results in **Fig. 4B** also suggest that the presence of hydrophobic residues (e.g., A, L, and I) interspaced between C-terminal positively charged residues are advantageous for Cas9-RNP resistance, as well as distancing the C-terminal positively charged residues from the "core" amphipathic cationic alpha helical region (e.g., CM18). Amongst all peptides tested in **Fig. 3**, FSD356 exhibited the highest GFP transduction efficiency (51%) in the presence of Cas9-RNP. The N-terminal segment of FSD356 is identical to that of FSD446, FSD357, FSD250, FSD296, FSD246, and FSD251. As shown in **Fig. 4C**, replacing the last three C-terminal residues of FSD356 (QAG) with three positively-charged arginine residues (RRR; see FSD357) resulted in a striking reduction in GFP transduction efficiency in the presence of Cas9-RNP – i.e., from 51% for FSD356 to a mere 4% for FSD357. In contrast, replacing the C-terminal RRR residues in FSD357 by *non-cationic* hydrophilic residues, including a negatively-charged aspartate (D) group, brought the GFP transduction efficiency in the presence of Cas9-RNP back up to 34%. Finally, replacing the C-terminal non-cationic hydrophilic

segment of FSD446 with a cationic segment (see FSD250) resulted in a drop in GFP transduction efficiency in the presence of Cas9-RNP down to 13%. Insertion of polar residues (e.g., Q) between the C-terminal positively-charged residues (e.g., FSD296) and/or increasing C-terminal positive charge density (e.g., FSD246) increased shuttle agent sensitivity to inhibition by Cas9-RNP (**Fig. 4C**). Strikingly, the shuttle agent FSD251, which contains three negatively-charged glutamate (E) residues in the peptide's C-terminal region, exhibited the least sensitivity to Cas9-RNP inhibition amongst the variants compared in **Fig. 4C**.

Of the peptides tested in **Fig. 3**, FSD174 was among those particularly sensitive to the inhibitory effect of Cas9-RNP, with its GFP transduction efficiency decreasing from 66% to 11% in the presence of Cas9-RNP. Structure-activity relationships relating to this inhibition were explored by repeating the above transduction experiments with shuttle agent variants sharing the same "core" amphipathic cationic alpha helical region as FSD174. As shown in **Fig. 4D**, shuttle agents having higher C-terminal positive charge densities (e.g., FSD189, FSD 174 and FSD187) were generally more sensitive to Cas9-RNP inhibition than shuttle agents having lower C-terminal positive charge densities. A comparison of the transduction efficiencies and structures of FSD168 vs FSD172, and FSD189 vs FSD 174, suggests that the presence of glycine/serine-rich residues increasing the distance of the C-terminal cationic residues from the N-terminal "core" amphipathic cationic alpha helical region may be advantageous for increased resistance to Cas9-RNP inhibition. Strikingly, FSD374 exhibited virtually the same GFP transduction efficiency in the presence or absence of Cas9-RNP (**Fig. 4D**). These results, which mirror those observed for FSD375 in **Fig 4A**, suggest that flanking the "core" amphipathic cationic alpha helical region with glycine/serine-rich residues is advantageous for resistance to Cas9-RNP inhibition.

The above transduction experiments were repeated with FSD10 and FSD375 in a Human Bronchial Epithelial cell line model of cystic fibrosis, CFF-16HBEge. As shown in **Fig. 5**, FSD375 exhibited greater resistance to Cas9-RNP inhibition than FSD10 in CFF-16HBEge cells as well.

Overall, the structure-function studies in this Example strongly suggest that reducing the cationic charge density (i.e., K/R residues per peptide segment length) in at least the C-terminal region of shuttle agents, for example by decreasing the number of positively charged residues per peptide segment length and/or interspacing the C-terminal positively charged residues with hydrophobic residues (e.g., A, L, and I), increases their resistance to Cas9-RNP inhibition (**Fig. 4** and **Fig. 5**). Furthermore, **Figs. 4 and 5** show that flanking a core amphiphilic cationic N-terminal segment of longer shuttle agents with non-cationic and/or negatively-charged hydrophilic residues may increase resistance to Cas9-sgRNA inhibition and likely to other nucleoprotein complexes.

Example 6: Shuttle agent-mediated delivery of functional Cas9/Cpf1-RNP complexes in HeLa cells

The ability of shuttle agents to deliver Cas9-RNP complexes intracellularly was measured indirectly in **Example 5** via the complexes' inhibitory effect on co-delivery of GFP. In the present Example, we assessed the ability of shuttle agents to deliver functional CRISPR Cas9-RNP or Cpf1-RNP complexes to the nucleus of target cells by measuring the phenotypic outcomes of successful genome editing. HeLa cells were incubated with different shuttle agents and Cas9-RNP or Cpf1-RNP targeting the gene encoding B2M ($\beta 2$ microglobulin), as described in **Example 1**. Six days post-delivery of Cas9-RNP or Cpf1-RNP complexes, genome editing efficiency was assessed by detection of cells lacking B2M expression (B2M knockout) by flow cytometry. **Fig. 6A-6E** show the results for delivery of functional Cas9- or Cpf1-sgRNA complexes by various synthetic shuttle agents. The results in **Fig. 6A-6E** generally show that the decrease in Cas9-RNP genome-editing efficiency, as compared to Cpf1-RNP, was generally smaller for shuttle agents having lower C-terminal cationic charge densities and/or having a core amphiphilic cationic segment flanked by one or more non-cationic hydrophilic residues.

Example 7: Shuttle agent-mediated delivery of functional Cas9/Cpf1/ABE-Cas9-sgRNA complexes in a Human Bronchial Epithelial cell line model of cystic fibrosis

A similar genome editing experiment as in **Example 6** was performed in the Human Bronchial Epithelial cell line model of cystic fibrosis, CFF-16HBEge. Genome editing results are shown in **Fig. 7**. Overall, lower genome editing efficiencies were observed in CFF-16HBEge cells as compared to HeLa cells, consistent with the refractory nature of lung epithelial cells. As seen in the results in HeLa cells in **Fig. 6A-6E**, shuttle agents transduced Cpf1-RNP complexes better than Cas9-RNP in CFF-16HBEge cells (**Fig. 7**). The shuttle agents FSD10, FSD322, FSD395, and FSD397 all exhibited greater than 10% genome editing efficiency with Cpf1-RNP, but failed to show any increase in genome editing over the non-treated (labelled) negative control with respect to Cas9-RNP. The only two shuttle agents amongst those tested that exhibited significant genome editing with Cas9-RNP as cargo were FSD374 and FSD375, which have similar structures of a core amphipathic cationic domain flanked by short segments of non-cationic hydrophilic residues.

Further transduction experiments in CFF-16HBEge cells were performed to compare the ability of shuttle agents to deliver functional Cas9-RNP genome editing versus ABE-Cas9-RNP base editing complexes. Variants of the shuttle agent FSD10 (**Fig. 8A**) were tested in parallel, and genome editing/base editing results as evaluated by next-generation sequencing (NGS) are shown in **Fig. 8B** and **8C** for Cas9-RNP and ABE-Cas9-RNP, respectively. Interestingly, delivery of ABE-Cas9-RNP base editing complex with the shuttle agent FSD375 resulted in significantly higher base editing efficiency (about 15%) over other shuttle agents tested (FSD10, FSD10-15, and FSD448) (**Fig. 8C**). Negative

control peptides consisting of the C-terminal cationic portion of FSD10 alone (“FSD10-Cter”) or flanked glycine/serine-rich linkers (“Linker-(FSD10-Cter)-Linker”) (**Fig. 8A**) did not exhibit any detectable genome editing or base editing as compared to non-treated cells (**Fig. 8B and 8C**).

5 **Example 8: Large-scale screening of candidate peptide shuttle agents for propidium iodide (PI) and GFP-NLS transduction activity**

A proprietary library of over 300 candidate peptide shuttle agents was screened in parallel for both propidium iodide (PI) and GFP-NLS transduction activity in HeLa cells using flow cytometry as generally described in **Example 1**. PI was used a cargo because it exhibits 20- to 30-fold enhanced
10 fluorescence and a detectable shift in maximum excitation/emission spectra *only after being bound to genomic DNA* – a property that makes it particularly suitable to distinguish endosomally-trapped cargo from endosomally-escaped cargo having access to the cytosolic/nuclear compartment. Thus, intracellular delivery and endosomal escape could both be measurable by flow cytometry since any PI that remained trapped in endosomes would not reach the nucleus and would exhibit neither the enhanced fluorescence
15 nor the spectra shift.

Due to the large number of peptides screened, negative controls were performed in parallel for each experimental batch and included a “no treatment” (NT) control in which the cells were not exposed to shuttle peptide or cargo, as well as a “cargo alone” control in which cells were exposed to the cargo in the absence of shuttle agent. Results are shown in **Fig. 9**, in which “transduction efficiency” refers to the
20 percentage of all viable cells that are positive for the cargo (PI or GFP-NLS). “Mean Delivery score” provides a further indication of the total amount of cargo that was delivered per cell, amongst all cargo-positive cells. Mean PI or GFP-NLS delivery score was calculated by multiplying the mean fluorescence intensity (of at least duplicate samples) measured for the viable PI+ or GFP+ cells by the mean percentage of viable PI+ or GFP+ cells, divided by 100,000 for GFP delivery or by 10,000 for PI delivery. The Mean
25 Delivery Scores for PI and GFP-NLS for each candidate shuttle agent was then normalized by dividing by the Mean Delivery Score for the “cargo alone” negative control performed in parallel for each experimental batch. Thus, the “Norm. Mean Delivery Score” in **Fig. 9** represents the fold-increase in Mean Delivery Score over the “cargo alone” negative control.

The batch-to-batch variation observed for the negative controls was relatively small for GFP-NLS
30 but was appreciably higher with PI as cargo. For example, the variation in transduction efficiency for the “cargo alone” negative control ranged from 0.4% to 1.3% for GFP-NLS and from 0.9% to 6.3% for PI. Furthermore, transduction efficiencies for several negative control peptides (i.e., peptides known to have low or no GFP transduction activity) tested in parallel (e.g., FSD174 Scramble; data not shown) sometimes gave lower transduction efficiencies for PI (but not for GFP-NLS) than the “cargo alone”

negative control, in some cases by as much as 5%, perhaps due to non-specific interactions between PI and the peptides. This phenomenon was not observed for GFP-NLS transduction experiments. The foregoing suggested that the shuttle agent transduction efficiencies at least for PI may be more appropriately compared to that of a negative control peptide rather than to the “cargo alone” condition.

5 Included amongst the candidate peptide shuttle agents in **Fig. 9** having a mean PI transduction efficiency of at least 20% were peptides having lengths of less than 20 residues: FSD390 (17 aa), FSD367 (19 aa), and FSD366 (18 aa). Also included amongst the candidate peptide shuttle agents having a mean PI transduction efficiency of at least 20% were peptides comprising either non-physiological amino acid analogs (e.g., FSD435, which corresponds to FSD395 except for lysine residues (K) being replaced with
10 L-2,4-diaminobutyric acid residues) or chemical modifications (e.g., FSD438, which corresponds to FSD10 except for an N-terminal octanoic acid modification; FSD436, which corresponds to FSD222 except for phenylalanine residues (F) being replaced with (2-naphthyl)-L-alanine residues; FSD171, which corresponds to FSD168 except having an N-terminal acetyl group and a C-terminal cysteamide group. These results confirm the robustness of the peptide shuttle agent platform technology to tolerate
15 the use of non-physiological amino acids or analogs thereof in place of physiological amino acids and/or chemical modifications.

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CLAIMS:

1. A composition comprising a nucleoprotein cargo for intracellular delivery and a synthetic peptide shuttle agent that is independent from, or is not covalently linked to, said nucleoprotein cargo, the synthetic peptide shuttle agent being a peptide comprising an amphipathic alpha-helical motif having both a positively-charged hydrophilic outer face and a hydrophobic outer face, wherein synthetic peptide shuttle agent increases cytosolic/nuclear delivery of said nucleoprotein cargo in eukaryotic cells as compared to in the absence of the synthetic peptide shuttle agent.
2. The composition of claim 1, wherein the nucleoprotein cargo is a deoxyribonucleoprotein (DNP) or ribonucleoprotein (RNP) cargo.
3. The composition of claim 1 or 2, wherein the nucleoprotein cargo is an RNA-guided nuclease, a Cas nuclease, such as a Cas type I, II, III, IV, V, or VI nuclease, or a variant thereof that lacking nuclease activity, a base editor, or a prime editor, a CRISPR-associated transposase, or a Cas-recombinase (e.g., recCas9).
4. The composition of any one of claims 1 to 3, the nucleoprotein cargo is Cpf1-RNP.
5. The composition of any one of claims 1 to 3, the nucleoprotein cargo is Cas9-RNP.
6. The composition of any one of claims 1 to 5, wherein the nucleoprotein cargo is not covalently linked or pre-complexed with a cell-penetrating peptide.
7. The composition of any one of claims 1 to 6, wherein the shuttle agent is:
 - (1) a peptide at least 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acids in length comprising
 - (2) an amphipathic alpha-helical motif having
 - (3) a positively-charged hydrophilic outer face, and a hydrophobic outer face,
 wherein at least five of the following parameters (4) to (15) are respected:
 - (4) the hydrophobic outer face comprises a highly hydrophobic core consisting of spatially adjacent L, I, F, V, W, and/or M amino acids representing 12 to 50% of the amino acids of the peptide, based on an open cylindrical representation of the alpha-helix having 3.6 residues per turn;
 - (5) the peptide has a hydrophobic moment (μ) of 3.5 to 11;
 - (6) the peptide has a predicted net charge of at least +3 or +4 at physiological pH;

- (7) the peptide has an isoelectric point (pI) of 8 to 13;
- (8) the peptide is composed of 35% to 65% of any combination of the amino acids: A, C, G, I, L, M, F, P, W, Y, and V;
- (9) the peptide is composed of 0% to 30% of any combination of the amino acids: N, Q, S, and T;
- (10) the peptide is composed of 35% to 85% of any combination of the amino acids: A, L, K, or R;
- (11) the peptide is composed of 15% to 45% of any combination of the amino acids: A and L, provided there being at least 5% of L in the peptide;
- (12) the peptide is composed of 20% to 45% of any combination of the amino acids: K and R;
- (13) the peptide is composed of 0% to 10% of any combination of the amino acids: D and E;
- (14) the difference between the percentage of A and L residues in the peptide (% A + L), and the percentage of K and R residues in the peptide (K + R), is less than or equal to 10%; and
- (15) the peptide is composed of 10% to 45% of any combination of the amino acids: Q, Y, W, P, I, S, G, V, F, E, D, C, M, N, T and H.
8. The composition of claim 7, wherein:
- (a) the shuttle agent respects at least six, at least seven, at least eight, at least nine, at least ten, at least eleven, or respects all of parameters (4) to (15);
- (b) the shuttle agent is a peptide having a minimum length of 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 amino acids, and a maximum length of 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 60, 65, 70, 80, 90, 100, 110, 120, 130, 140, or 150 amino acids;
- (c) said amphipathic alpha-helical motif has a hydrophobic moment (μ) between a lower limit of 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7.0, and an upper limit of 9.5, 9.6, 9.7, 9.8, 9.9, 10.0, 10.1, 10.2, 10.3, 10.4, 10.5, 10.6, 10.7, 10.8, 10.9, or 11.0;
- (d) said amphipathic alpha-helical motif comprises a positively-charged hydrophilic outer face comprising: (i) at least two, three, or four adjacent positively-charged K and/or R residues upon helical wheel projection; and/or (ii) a segment of six adjacent residues comprising three to five K and/or R residues upon helical wheel projection, based on an alpha helix having angle of rotation between consecutive amino acids of 100 degrees and/or an alpha-helix having 3.6 residues per turn;

- (e) said amphipathic alpha-helical motif comprises a hydrophobic outer face comprising: (i) at least two adjacent L residues upon helical wheel projection; and/or (ii) a segment of ten adjacent residues comprising at least five hydrophobic residues selected from: L, I, F, V, W, and M, upon helical wheel projection, based on an alpha helix having angle of rotation between consecutive amino acids of 100 degrees and/or an alpha-helix having 3.6 residues per turn;
- (f) said hydrophobic outer face comprises a highly hydrophobic core consisting of spatially adjacent L, I, F, V, W, and/or M amino acids representing from 12.5%, 13%, 13.5%, 14%, 14.5%, 15%, 15.5%, 16%, 16.5%, 17%, 17.5%, 18%, 18.5%, 19%, 19.5%, or 20%, to 25%, 30%, 35%, 40%, or 45% of the amino acids of the shuttle agent;
- (g) the shuttle agent has a hydrophobic moment (μ) between a lower limit of 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7.0, and an upper limit of 9.5, 9.6, 9.7, 9.8, 9.9, 10.0, 10.1, 10.2, 10.3, 10.4, or 10.5;
- (h) the shuttle agent has a predicted net charge of between +3, +4, +5, +6, +7, +8, +9, to +10, +11, +12, +13, +14, or +15;
- (i) the shuttle agent has a predicted pI of 10 to 13; or
- (j) any combination of (a) to (i).
9. The composition of claim 7 or 8, wherein said shuttle agent respects at least one, at least two, at least three, at least four, at least five, at least six, or all of the following parameters:
- (8) the shuttle agent is composed of 36% to 64%, 37% to 63%, 38% to 62%, 39% to 61%, or 40% to 60% of any combination of the amino acids: A, C, G, I, L, M, F, P, W, Y, and V;
- (9) the shuttle agent is composed of 1% to 29%, 2% to 28%, 3% to 27%, 4% to 26%, 5% to 25%, 6% to 24%, 7% to 23%, 8% to 22%, 9% to 21%, or 10% to 20% of any combination of the amino acids: N, Q, S, and T;
- (10) the shuttle agent is composed of 36% to 80%, 37% to 75%, 38% to 70%, 39% to 65%, or 40% to 60% of any combination of the amino acids: A, L, K, or R;
- (11) the shuttle agent is composed of 15% to 40%, 20% to 40%, 20 to 35%, or 20 to 30% of any combination of the amino acids: A and L;
- (12) the shuttle agent is composed of 20% to 40%, 20 to 35%, or 20 to 30% of any combination of the amino acids: K and R;
- (13) the shuttle agent is composed of 5 to 10% of any combination of the amino acids: D and E;

- (14) the difference between the percentage of A and L residues in the shuttle agent (% A + L), and the percentage of K and R residues in the shuttle agent (K + R), is less than or equal to 9%, 8%, 7%, 6%, or 5%; and
- (15) the shuttle agent is composed of 15 to 40%, 20% to 35%, or 20% to 30% of any combination of the amino acids: Q, Y, W, P, I, S, G, V, F, E, D, C, M, N, T, and H.

10. The composition of any one of claims 1 to 9, wherein said shuttle agent comprises a histidine-rich domain, optionally wherein the histidine-rich domain is:

- (i) positioned towards the N terminus and/or towards the C terminus of the shuttle agent;
- (ii) is a stretch of at least 3, at least 4, at least 5, or at least 6 amino acids comprising at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, or at least 90% histidine residues; and/or comprises at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, or at least 9 consecutive histidine residues; or
- (iii) both (i) and (ii).

11. The composition of any one of claims 1 to 10, wherein said shuttle agent comprises a flexible linker domain rich in serine and/or glycine residues (e.g., separating N-terminal and a C-terminal segments of the shuttle agent; or positioned N- and/or C-terminal of a central amphipathic cationic alpha helical domain).

12. The composition of any one of claims 1 to 11, wherein said shuttle agent comprises or consists of the amino acid sequence of:

- (a) [X1]-[X2]-[linker]-[X3]-[X4] (Formula 1);
- (b) [X1]-[X2]-[linker]-[X4]-[X3] (Formula 2);
- (c) [X2]-[X1]-[linker]-[X3]-[X4] (Formula 3);
- (d) [X2]-[X1]-[linker]-[X4]-[X3] (Formula 4);
- (e) [X3]-[X4]-[linker]-[X1]-[X2] (Formula 5);
- (f) [X3]-[X4]-[linker]-[X2]-[X1] (Formula 6);
- (g) [X4]-[X3]-[linker]-[X1]-[X2] (Formula 7);
- (h) [X4]-[X3]-[linker]-[X2]-[X1] (Formula 8);
- (i) [linker]-[X1]-[X2]-[linker] (Formula 9);
- (j) [linker]-[X2]-[X1]-[linker] (Formula 10);
- (k) [X1]-[X2]-[linker] (Formula 11);
- (l) [X2]-[X1]-[linker] (Formula 12);

- (m) **[linker]-[X1]-[X2]** (Formula 13);
- (n) **[linker]-[X2]-[X1]** (Formula 14);
- (o) **[X1]-[X2]** (Formula 15); or
- (p) **[X2]-[X1]** (Formula 16),

5 wherein:

[X1] is selected from: $2[\Phi]-1[+]-2[\Phi]-1[\zeta]-1[+]$; $2[\Phi]-1[+]-2[\Phi]-2[+]$; $1[+]-1[\Phi]-1[+]-2[\Phi]-1[\zeta]-1[+]$; and $1[+]-1[\Phi]-1[+]-2[\Phi]-2[+]$;

[X2] is selected from: $-2[\Phi]-1[+]-2[\Phi]-2[\zeta]$; $-2[\Phi]-1[+]-2[\Phi]-2[+]$; $-2[\Phi]-1[+]-2[\Phi]-1[+]-1[\zeta]$; $-2[\Phi]-1[+]-2[\Phi]-1[\zeta]-1[+]$; $-2[\Phi]-2[+]-1[\Phi]-2[+]$; $-2[\Phi]-2[+]-1[\Phi]-2[\zeta]$; $-2[\Phi]-2[+]-1[\Phi]-1[+]-1[\zeta]$; and $-2[\Phi]-2[+]-1[\Phi]-1[\zeta]-1[+]$;

[X3] is selected from: $-4[+]-A$; $-3[+]-G-A$; $-3[+]-A-A$; $-2[+]-1[\Phi]-1[+]-A$; $-2[+]-1[\Phi]-G-A$; $-2[+]-1[\Phi]-A-A$; or $-2[+]-A-1[+]-A$; $-2[+]-A-G-A$; $-2[+]-A-A-A$; $-1[\Phi]-3[+]-A$; $-1[\Phi]-2[+]-G-A$; $-1[\Phi]-2[+]-A-A$; $-1[\Phi]-1[+]-1[\Phi]-1[+]-A$; $-1[\Phi]-1[+]-1[\Phi]-G-A$; $-1[\Phi]-1[+]-1[\Phi]-A-A$; $-1[\Phi]-1[+]-A-1[+]-A$; $-1[\Phi]-1[+]-A-G-A$; $-1[\Phi]-1[+]-A-A-A$; $-A-1[+]-A-1[+]-A$; $-A-1[+]-A-G-A$; and $-A-1[+]-A-A-A$;

[X4] is selected from: $-1[\zeta]-2A-1[+]-A$; $-1[\zeta]-2A-2[+]$; $-1[+]-2A-1[+]-A$; $-1[\zeta]-2A-1[+]-1[\zeta]-A-1[+]$; $-1[\zeta]-A-1[\zeta]-A-1[+]$; $-2[+]-A-2[+]$; $-2[+]-A-1[+]-A$; $-2[+]-A-1[+]-1[\zeta]-A-1[+]$; $-2[+]-1[\zeta]-A-1[+]$; $-1[+]-1[\zeta]-A-1[+]-A$; $-1[+]-1[\zeta]-A-2[+]$; $-1[+]-1[\zeta]-A-1[+]-1[\zeta]-A-1[+]$; $-1[+]-2[\zeta]-A-1[+]$; $-1[+]-2[\zeta]-2[+]$; $-1[+]-2[\zeta]-1[+]-A$; $-1[+]-2[\zeta]-1[+]-1[\zeta]-A-1[+]$; $-1[+]-2[\zeta]-1[\zeta]-A-1[+]$; $-3[\zeta]-2[+]$; $-3[\zeta]-1[+]-A$; $-3[\zeta]-1[+]-1[\zeta]-A-1[+]$; $-1[\zeta]-2A-1[+]-A$; $-1[\zeta]-2A-2[+]$; $-1[\zeta]-2A-1[+]-1[\zeta]-A-1[+]$; $-2[+]-A-1[+]-A$; $-2[+]-1[\zeta]-1[+]-A$; $-1[+]-1[\zeta]-A-1[+]-A$; $-1[+]-2A-1[+]-1[\zeta]-A-1[+]$; and $-1[\zeta]-A-1[\zeta]-A-1[+]$; and

[linker] is selected from: $-Gn$; $-Sn$; $-(GnSn)n$; $-(GnSn)nGn$; $-(GnSn)nSn$; $-(GnSn)nGn(GnSn)n$; and $-(GnSn)nSn(GnSn)n$;

25 wherein:

[Φ] is an amino acid which is: Leu, Phe, Trp, Ile, Met, Tyr, or Val, preferably Leu, Phe, Trp, or Ile;

[+] is an amino acid which is: Lys or Arg;

[ζ] is an amino acid which is: Gln, Asn, Thr, or Ser;

A is the amino acid Ala;

30 **G** is the amino acid Gly;

S is the amino acid Ser; and

n is an integer from 1 to 20, 1 to 19, 1 to 18, 1 to 17, 1 to 16, 1 to 15, 1 to 14, 1 to 13, 1 to 12, 1 to 11, 1 to 10, 1 to 9, 1 to 8, 1 to 7, 1 to 6, 1 to 5, 1 to 4, or 1 to 3.

13. The composition of any one of claims 1 to 12, wherein the shuttle agent comprises or consists of:
- (i) the amino acid sequence any one of **SEQ ID NOs: 1 to 50, 58 to 78, 80 to 107, 109 to 139, 141 to 146, 149 to 161, 163 to 169, 171, 174 to 234, 236 to 240, 242 to 260, 262 to 285, 287 to 294, 296 to 300, 302 to 308, 310, 311, 313 to 324, 326 to 332, 338 to 342, 344, 346, 348, 352, 355, 356, 358 to 360, 362, 363, 366, 369, 370, or 379;**
 - (ii) an amino acid sequence that differs from any one of **SEQ ID NOs: 1 to 50, 58 to 78, 80 to 107, 109 to 139, 141 to 146, 149 to 161, 163 to 169, 171, 174 to 234, 236 to 240, 242 to 260, 262 to 285, 287 to 294, 296 to 300, 302 to 308, 310, 311, 313 to 324, 326 to 332, 338 to 342, 344, 346, 348, 352, 355, 356, 358 to 360, 362, 363, 366, 369, 370 or 379** by no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acids (e.g., excluding any linker domains);
 - (iii) an amino acid sequence that is at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to any one of **SEQ ID NOs: 1 to 50, 58 to 78, 80 to 107, 109 to 139, 141 to 146, 149 to 161, 163 to 169, 171, 174 to 234, 236 to 240, 242 to 260, 262 to 285, 287 to 294, 296 to 300, 302 to 308, 310, 311, 313 to 324, 326 to 332, 338 to 342, 344, 346, 348, 352, 355, 356, 358 to 360, 362, 363, 366, 369, 370 or 379** (e.g., calculated excluding any linker domains);
 - (iv) an amino acid sequence that differs from any one of **SEQ ID NOs: 1 to 50, 58 to 78, 80 to 107, 109 to 139, 141 to 146, 149 to 161, 163 to 169, 171, 174 to 234, 236 to 240, 242 to 260, 262 to 285, 287 to 294, 296 to 300, 302 to 308, 310, 311, 313 to 324, 326 to 332, 338 to 342, 344, 346, 348, 352, 355, 356, 358 to 360, 362, 363, 366, 369, 370 or 379** by only conservative amino acid substitutions (e.g., by no more than no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 conservative amino acid substitutions, preferably excluding any linker domains), wherein each conservative amino acid substitution is selected from an amino acid within the same amino acid class, the amino acid class being: Aliphatic: G, A, V, L, and I; Hydroxyl or sulfur/selenium-containing: S, C, U, T, and M; Aromatic: F, Y, and W; Basic: H, K, and R; Acidic and their amides: D, E, N, and Q; or
 - (v) any combination of (i) to (iv).
14. The composition of any one of claims 1 to 13, wherein the shuttle agent comprises or consists of:
- (a) a fragment of a parent shuttle agent as defined in any one of claims 7 to 13, wherein the fragment retains cargo transduction activity and comprises an amphipathic alpha-helical

motif having both a positively-charged hydrophilic outer face and a hydrophobic outer face,
or

- (b) a variant of a parent shuttle agent as defined in any one of claims 7 to 13, wherein the variant retains cargo transduction activity and differs (or differs only) from the parent shuttle agent by having a reduced C-terminal positive charge density relative to the parent shuttle agent (e.g., by substituting one or more cationic residues, such as K/R, with non-cationic residues, preferably non-cationic hydrophilic residues; and/or by engineering hydrophobic residues (e.g., A, V, L, I, F, or W) between two proximal cationic residues;

wherein the fragment or variant has increased resistance to inhibition by the nucleoprotein cargo and/or to the presence of DNA and/or RNA, and/or has increased transduction activity for the nucleoprotein cargo.

15. The composition of claim 14, wherein the fragment or variant comprises or consists of a C-terminal truncation of the parent shuttle agent.

16. The composition of claim 14 or 15, wherein the fragment or variant comprises an amphipathic alpha-helical motif having both a positively-charged hydrophilic outer face and a hydrophobic outer face, which is flanked by or at least by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 non-cationic hydrophilic residues, such that the fragment or variant retains cargo transduction activity and/or has increased resistance to inhibition by the nucleoprotein cargo.

17. The composition of any one of claims 1 to 16, wherein shuttle agent comprises or consists of a peptide less than 20 amino acids in length.

18. The composition of any one of claims 1 to 17, wherein the shuttle agent comprises a helical region comprising an amphipathic helix harboring:

- a cluster of hydrophobic amino acid residues on one side of the helix defining a hydrophobic angle of 140° to 280° in Schiffer-Edmundson's wheel representation, and
- a cluster of positively charged residues on the other side of the helix defining a positively charged angle of 40° to 160° in Schiffer-Edmundson's wheel representation.

19. The composition of claim 18, wherein the hydrophobic angle is 160° to 260° in Schiffer-Edmundson's wheel representation.

20. The composition of claim 18, wherein the hydrophobic angle is 180° to 240° in Schiffer-Edmundson's wheel representation.
21. The composition of any one of claims 18 to 20, wherein the positively charged angle is 40° to 140° in Schiffer-Edmundson's wheel representation.
22. The composition of any one of claims 18 to 20, wherein the positively charged angle is 60° to 140° in Schiffer-Edmundson's wheel representation.
23. The composition of any one of claims 18 to 20, wherein the positively charged angle is 60° to 120° in Schiffer-Edmundson's wheel representation.
24. The composition of any one of claims 18 to 23, wherein at least 20%, 30%, 40%, or 50% of the residues in the hydrophobic cluster are hydrophobic residues.
25. The composition of claim 24, wherein the hydrophobic residues are selected from the group consisting of phenylalanine, isoleucine, tryptophan, leucine, valine, methionine, tyrosine, cysteine, glycine, and alanine.
26. The composition of claim 24, wherein the hydrophobic residues are selected from the group consisting of phenylalanine, isoleucine, tryptophan, and leucine.
27. The composition of any one of claims 18 to 26, wherein at least 20%, 30%, 40%, or 50% of the residues in the positively charged cluster are positively charged residues.
28. The composition of claim 27, wherein the positively charged residues are selected from the group consisting of lysine, arginine, and histidine.
29. The composition of claim 28, wherein the positively charged residues are selected from the group consisting of lysine and arginine.
30. The composition of any one of 18 to 29, wherein the synthetic peptide shuttle agent is at least 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, or 19 amino acids in length.

31. The composition of any one of claims 1 to 30, wherein the synthetic peptide shuttle agent has a hydrophobic moment (μH) of at least 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, 5.1, 5.2, 5.3, 5.4, or 5.5.
- 5 32. The composition of any one of claims 1 to 31, wherein the shuttle agent comprises or consists of a variant of the synthetic peptide shuttle agent, the variant being identical to the synthetic peptide shuttle agent as defined in any one of claims 1 or 7 to 31, except having at least one amino acid being replaced with a corresponding synthetic amino acid having a side chain of similar physiochemical properties (e.g., structure, hydrophobicity, or charge) as the amino acid being replaced, wherein the variant increases
- 10 cytosolic/nuclear delivery of said cargo in eukaryotic cells as compared to in the absence of the synthetic peptide shuttle agent, preferably wherein the synthetic amino acid replacement:
- (a) replaces a basic amino acids with any one of: α -aminoglycine, α,γ -diaminobutyric acid, ornithine, α,β -diaminopropionic acid, 2,6-diamino-4-hexynoic acid, β -(1-piperazinyl)-alanine, 4,5-dehydro-lysine, δ -hydroxylysine, ω,ω -dimethylarginine, homoarginine, ω,ω' -dimethylarginine, ω -methylarginine, β -(2-quinolyl)-alanine, 4-aminopiperidine-4-carboxylic acid, α -methylhistidine, 2,5-diiodohistidine, 1-methylhistidine, 3-methylhistidine, spinacine, 4-aminophenylalanine, 3-aminotyrosine, β -(2-pyridyl)-alanine, or β -(3-pyridyl)-alanine;
 - (b) replaces a non-polar (hydrophobic) amino acid with any one of: dehydro-alanine, β -fluoroalanine, β -chloroalanine, β -iodoalanine, α -aminobutyric acid, α -aminoisobutyric acid, β -cyclopropylalanine, azetidine-2-carboxylic acid, α -allylglycine, propargylglycine, tert-butylalanine, β -(2-thiazolyl)-alanine, thiaproline, 3,4-dehydroproline, tert-butylglycine, β -cyclopentylalanine, β -cyclohexylalanine, α -methylproline, norvaline, α -methylvaline, penicillamine, β , β -dicyclohexylalanine, 4-fluoroproline, 1-aminocyclopentanecarboxylic acid, pipecolic acid, 4,5-dehydroleucine, allo-isoleucine, norleucine, α -methylleucine, cyclohexylglycine, cis-octahydroindole-2-carboxylic acid, β -(2-thienyl)-alanine, phenylglycine, α -methylphenylalanine, homophenylalanine, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid, β -(3-benzothienyl)-alanine, 4-nitrophenylalanine, 4-bromophenylalanine, 4-tert-butylphenylalanine, α -methyltryptophan, β -(2-naphthyl)-alanine, β -(1-naphthyl)-alanine, 4-iodophenylalanine, 3-fluorophenylalanine, 4-fluorophenylalanine, 4-methyltryptophan, 4-chlorophenylalanine, 3,4-dichloro-phenylalanine, 2,6-difluoro-phenylalanine, n-in-methyltryptophan, 1,2,3,4-tetrahydronorharman-3-carboxylic acid, β,β -diphenylalanine, 4-methylphenylalanine, 4-phenylphenylalanine, 2,3,4,5,6-pentafluoro-phenylalanine, or 4-benzoylphenylalanine;

- (c) replaces a polar, uncharged amino acid with any one of: β -cyanoalanine, β -ureidoalanine, homocysteine, allo-threonine, pyroglutamic acid, 2-oxothiazolidine-4-carboxylic acid, citrulline, thiocitrulline, homocitrulline, hydroxyproline, 3,4-dihydroxyphenylalanine, β -(1,2,4-triazol-1-yl)-alanine, 2-mercaptohistidine, β -(3,4-dihydroxyphenyl)-serine, β -(2-thienyl)-serine, 4-azidophenylalanine, 4-cyanophenylalanine, 3-hydroxymethyltyrosine, 3-iodotyrosine, 3-nitrotyrosine, 3,5-dinitrotyrosine, 3,5-dibromotyrosine, 3,5-diiodotyrosine, 7-hydroxy-1,2,3,4-tetrahydroiso-quinoline-3-carboxylic acid, 5-hydroxytryptophan, thyronine, β -(7-methoxycoumarin-4-yl)-alanine, or 4-(7-hydroxy-4-coumarinyl)-aminobutyric acid; and/or
- (d) replaces an acidic amino acid with any one of: γ -hydroxyglutamic acid, γ -methyleneglutamic acid, γ -carboxyglutamic acid, α -aminoadipic acid, 2-aminoheptanedioic acid, α -aminosuberic acid, 4-carboxyphenylalanine, cysteic acid, 4-phosphonophenylalanine, or 4-sulfomethylphenylalanine.
33. The composition of any one of claims 1 to 32, wherein the shuttle agent:
- does not comprise a cell penetrating domain (CPD), a cell-penetrating peptide (CPP), or a protein transduction domain (PTD); or
 - does not comprise a CPD fused to an endosome leakage domain (ELD).
34. The composition of any one of claims 1 to 32, wherein the shuttle agent comprises an endosome leakage domain (ELD) and/or a cell penetrating domain (CPD).
35. The composition of any one of claims 33 or 34, wherein:
- (i) said ELD is or is from: an endosomolytic peptide; an antimicrobial peptide (AMP); a linear cationic alpha-helical antimicrobial peptide; a Cecropin-A/Melittin hybrid (CM) peptide; pH-dependent membrane active peptide (PAMP); a peptide amphiphile; a peptide derived from the N terminus of the HA2 subunit of influenza hemagglutinin (HA); CM18; Diphtheria toxin T domain (DT); GALA; PEA; INF-7; LAH4; HGP; H5WYG; HA2; EB1; VSVG; Pseudomonas toxin; melittin; KALA; JST-1; C(LLKK)₃C; G(LLKK)₃G; or any combination thereof;
 - (ii) said CPD is or is from: a cell-penetrating peptide or the protein transduction domain from a cell-penetrating peptide; TAT; PTD4; Penetratin; pVEC; M918; Pep-1; Pep-2; Xentry; arginine stretch; transportan; SynB1; SynB3; or any combination thereof; or
 - (iii) both (i) and (ii).

36. The composition of any one of claims 1 to 35, wherein the shuttle agent is a cyclic peptide and/or comprises one or more D-amino acids.
- 5 37. The composition of any one of claims 1 to 36, wherein the shuttle agent increases the transduction efficiency and/or total amount of nucleoprotein cargo delivered intracellularly in the eukaryotic cells by at least 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, or 10-fold over a corresponding negative control lacking said shuttle agent.
- 10 38. The composition of any one of claims 1 to 37, wherein the shuttle agent further comprises a chemical modification to one or more amino acids, wherein the chemical modification does not destroy the transduction activity of the synthetic peptide shuttle agent.
39. The composition of claim 38, wherein the chemical modification is at the N and/or C terminus of
15 the shuttle agent.
40. The composition of claim 38 or 39, wherein the chemical modification is the addition of an acetyl group (e.g., an N-terminal acetyl group), a cysteamide group (e.g., a C-terminal cysteamide group), or a fatty acid (e.g., C4-C16 fatty acid, preferably N-terminal).
- 20 41. The composition of any one of claims 1 to 40, wherein the concentration of the nucleoprotein cargo and/or the synthetic peptide shuttle agent in the composition is at least 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 μM .
- 25 42. The composition as defined in any one of claims 1 to 41:
- (a) for use in increasing the transduction efficiency of the nucleoprotein cargo to the cytosolic/nuclear compartment of eukaryotic cells;
 - (b) for use in genome editing, base editing, or prime editing in eukaryotic cells;
 - 30 (c) for use in modulating gene expression in the eukaryotic cells;
 - (d) for use in therapy, wherein the nucleoprotein cargo binds to a therapeutic target in the eukaryotic cells;
 - (e) for use in delivering a non-therapeutic nucleoprotein cargo as a diagnostic agent;
 - (f) for use in the manufacture of a medicament or diagnostic agent;

- (g) for use in treating cancer (e.g., skin cancer, basal cell carcinoma, nevoid basal cell carcinoma syndrome), inflammation or an inflammation-related disease (e.g., psoriasis, atopic dermatitis, ulcerative colitis, urticaria, dry eye disease, dry or wet age-related macular degeneration, digital ulcers, actinic keratosis, idiopathic pulmonary fibrosis), pain (e.g., chronic or acute), or a disease affecting the lungs (e.g., cystic fibrosis, asthma, chronic obstructive pulmonary disease (COPD), or idiopathic pulmonary fibrosis); or
- (h) any combination of (a) to (g).

43. The composition of any one of claims 1 to 41, or the composition for use of claim 42, wherein the eukaryotic cells are animal cells, mammalian cells, human cells, stem cells, primary cells, immune cells, T cells, NK cells, dendritic cells, epithelial cells, skin cells, gastrointestinal cells, lung cells, or ocular cells.

44. A method for the use as defined in claim 42, the method comprising:
- (a) providing a nucleoprotein cargo for intracellular delivery in a population of eukaryotic cells;
- (b) providing a synthetic peptide shuttle agent that is independent from, or is not covalently linked to, said nucleoprotein cargo;
- (c) contacting the eukaryotic cells with the nucleoprotein cargo in the presence of the synthetic peptide shuttle agent at a concentration sufficient to increase the transduction efficiency and/or cytosolic/nuclear delivery of the nucleoprotein cargo, as compared to in the absence of said synthetic peptide shuttle agent,
- wherein the nucleoprotein cargo binds to an intracellular target, thereby effecting said use.

45. The method of claim 44, which is an *in vitro* method (e.g., for a therapeutic and/or diagnostic purpose).

46. The method of claim 44, which is an *in vivo* method (e.g., for therapeutic and/or diagnostic purpose).

47. The method of any one of claims 44 to 46, wherein:
- (i) the nucleoprotein cargo is as defined in any one of claims 1 to 6;
- (ii) the synthetic peptide shuttle agent is as defined in any one of claims 1 or 7 to 23;
- (iii) the eukaryotic cells are contacted a concentration of the cargo and/or the synthetic peptide shuttle agent as defined in claim 24;

- (iv) the method is for a use as defined in claim 25;
- (v) the eukaryotic cells are as defined in claim 26; or
- (vi) any combination of (i) to (v).

5 48. A method for cargo transduction, the method comprising contacting target eukaryotic cells with said cargo and a concentration of a synthetic peptide shuttle agent sufficient to increase the transduction efficiency of said cargo, as compared to in the absence of said synthetic peptide shuttle agent, wherein said synthetic peptide shuttle agent is a peptide less than 20 amino acids in length, and wherein the shuttle agent and cargo are not covalently bound at the time of transduction across the plasma membrane.

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49. The method of claim 48, wherein the synthetic peptide shuttle agent comprises a helical region comprising an amphipathic helix harboring:

- a cluster of hydrophobic amino acid residues on one side of the helix defining a hydrophobic angle of 140° to 280° in Schiffer-Edmundson's wheel representation, and
- 15 – a cluster of positively charged residues on the other side of the helix defining a positively charged angle of 40° to 160° in Schiffer-Edmundson's wheel representation.

50. The method of claim 49, wherein the hydrophobic angle is 160° to 260° in Schiffer-Edmundson's wheel representation.

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51. The method of claim 49, wherein the hydrophobic angle is 180° to 240° in Schiffer-Edmundson's wheel representation.

52. The method of any one of claims 49 to 51, wherein the positively charged angle is 40° to 140° in Schiffer-Edmundson's wheel representation.

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53. The method of any one of claims 49 to 51, wherein the positively charged angle is 60° to 140° in Schiffer-Edmundson's wheel representation.

54. The method of any one of claims 49 to 51, wherein the positively charged angle is 60° to 120° in Schiffer-Edmundson's wheel representation.

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55. The method of any one of claims 49 to 54, wherein at least 20%, 30%, 40%, or 50% of the residues in the hydrophobic cluster are hydrophobic residues.

56. The method of claim 55, wherein the hydrophobic residues are selected from the group consisting of phenylalanine, isoleucine, tryptophan, leucine, valine, methionine, tyrosine, cysteine, glycine, and alanine.
57. The method of claim 55, wherein the hydrophobic residues are selected from the group consisting of phenylalanine, isoleucine, tryptophan, and/or leucine.
58. The method of any one of claims 49 to 57, wherein at least 20%, 30%, 40%, or 50% of the residues in the positively charged cluster are positively charged residues.
59. The method of any one of claims 49 to 58, wherein the positively charged residues are selected from the group consisting of lysine, arginine, and histidine.
60. The method of any one of claims 49 to 58, wherein the positively charged residues are selected from the group consisting of lysine and arginine.
61. The method of any one of claims 49 to 60, wherein the synthetic peptide shuttle agent is at least 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, or 19 amino acids in length.
62. The method of any one of claims 49 to 61, wherein the synthetic peptide shuttle agent has a hydrophobic moment (μH) of at least 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, 5.1, 5.2, 5.3, 5.4, or 5.5.
63. The method of any one of claims 49 to 62, wherein the cargo is a polypeptide, peptide, nucleoprotein (e.g., as defined in any one of claims 1 to 6), small molecule, oligonucleotide, or oligonucleotide analog (e.g., non-anionic oligonucleotide analog).
64. A synthetic peptide which is the shuttle agent as defined in any one of claims 1 to 63.
65. The synthetic peptide of claim 64 for use in therapy and/or in cargo transduction (e.g., polypeptide, peptide, nucleoprotein (e.g., as defined in any one of claims 1 to 6), small molecule, oligonucleotide, or oligonucleotide analog (e.g., non-anionic oligonucleotide analog) in eukaryotic cells, wherein the shuttle agent is used at concentration sufficient to increase the transduction efficiency of said

cargo, as compared to in the absence of said synthetic peptide shuttle agent, and wherein the synthetic peptide shuttle agent and cargo are not covalently bound.

66. Use of the synthetic peptide shuttle agent as defined in any one of claims 1 to 63 for the
5 manufacture of a medicament (e.g., for treating a disease as defined in claim 42).
67. A composition comprising the synthetic peptide shuttle agent of claim 64 and a suitable excipient.
68. A synthetic peptide shuttle agent for use in, or suitable for use in, the delivery of non-anionic
10 cargoes across mucus-producing membranes (e.g., airway epithelium), the synthetic peptide shuttle agent comprising or consisting essentially of a central core amphipathic alpha helical region having shuttle agent activity, flanked N- and C-terminally by flexible linker domains, wherein one or both of the flexible linker domains comprises or consists essentially of a sufficient number of non-cationic hydrophilic residues such that cargo transduction activity across mucus-producing membranes of the synthetic peptide
15 shuttle agent is increased relative to that of the central core amphipathic alpha helical region lacking the flexible linker domains.
69. The synthetic peptide shuttle agent of claim 68, wherein the central core amphipathic alpha helical region:
20 (a) is an endosomolytic peptide;
(b) is at least 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, or 19 amino acids in length;
(c) is a fragment of a parent shuttle agent as defined in claim 14(a) or 15;
(d) is an amphipathic helix as defined in any one of claims 18 to 29 or 49 to 60;
(e) has a hydrophobic moment (μH) of at least 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0,
25 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, 5.1, 5.2, 5.3, 5.4, or 5.5; or
(f) any combination of (a) to (e).
70. The synthetic peptide shuttle agent of claim 68 or 69, wherein the non-cationic hydrophilic residues comprise or consist essentially of glycine, serine, aspartate, glutamate, histidine, tyrosine,
30 threonine, cysteine, asparagine, glutamine, or any combination thereof.
71. The synthetic peptide shuttle agent of any one of claims 68 to 70, wherein the flexible linker domain is as defined in claim 12.

72. The synthetic peptide shuttle agent of any one of claims 68 to 71 for the use as defined in claim 42 or 43.